REMARKS

Applicant has amended the specification to update related application information as requested by the Examiner. Applicant has also added to the Brief Description of the Drawings sequence identifiers of the sequences in Figure 13 as requested by the Examiner. A marked up version of the amended paragraphs in the specification, with amendments indicated by bracketing for deletions and underlining for additions, is attached hereto as Exhibit A. No new matter has been added by these amendments.

Claims 8-24 were pending in this application. Claims 13, 16 and 18 have been canceled without prejudice to Applicant's right to pursue the subject matter of any canceled claims in subsequent applications. Claims 8-12, 14-15, 17 and 19-24 have been amended to more particularly point out and distinctly claim the subject matter which Applicant regards as the invention, and new claims 25-26 have been added. Applicant retains the right to pursue the canceled subject matter in subsequent applications. Claims 8-12, 14-15, 17 and 19-26 are fully supported by the specification as originally filed, such that the above-made amendments do not constitute new matter under 35 U.S.C. § 132. Accordingly, claims 8-12, 14-15, 17 and 19-26 will be pending upon entry of this amendment. A marked up version of the amended claims, with amendments indicated by bracketing for deletions and underlining for additions, is attached hereto as Exhibit B.

The Rejections Under 35 U.S.C. § 112, Second Paragraph Should Be Withdrawn

Claims 8-12, 15, 17 and 20-22 stand rejected under 35 U.S.C. §112, second paragraph as allegedly being indefinite. Claims 8-15, 17 and 19-24 have been amended to more particularly point out and distinctly claim the subject matter which Applicant regard as the invention. Each point raised by the Examiner in Item No. 5 of the July 3, 2001 Office Action (at page 3) has been addressed in the above amendments as suggested by the Examiner, and in accordance with the requirements under 35 U.S.C. §112. As such, Applicant respectfully

submits that the rejections of claims 8-12, 15, 17 and 20-22 under 35 U.S.C. §112, second paragraph have been obviated and overcome; therefore, Applicant respectfully requests that the rejections under 35 U.S.C. §112, second paragraph be withdrawn.

The Rejections Under 35 U.S.C. § 112, First Paragraph Should Be Withdrawn

Claims 8-24 stand rejected under 35 U.S.C. §112, first paragraph as allegedly containing subject matter not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention. More specifically, the Examiner states that "the claims as written read broadly on use of any nucleotide which hybridizes to SEQ ID NO:19, of any specified length or oligonucleotide composition, and further wherein the claimed treatment is of any organism for any disorder related to bcl-2 expression" (July 3, 2001 Office Action at page 4). Further, the Examiner contends that, although successful use of bcl-2 antisense compounds are found in the post-art, "such examples do not provide a representative number of species for enablement of any oligonucleotide which could hybridize to SEQ ID NO:19 to be considered a suitable candidate as a therapeutic agent for any bcl-2 related disorder in any whole organism" (July 3, 2001 Office Action at page 5).

In response, Applicant has amended all independent claims to relate to use of anticode oligomers of 10-40 bases in length for targeting human bcl-2. Support for these amendments can be found, for example, at page 11, lines 31-35 and of the original specification. Applicant respectfully submits that the above-made amendments address the Examiner's concerns regarding the use of "any oligonucleotide" for use in "any organism" such that the teachings of the original specification, coupled with the state of the art at the time of filing, provides the skilled artisan with sufficient guidance to make and use the claimed invention.

Briefly, the test for enablement is whether one skilled in the art could make and use the claimed invention, without undue experimentation, from the disclosure in the patent

specification coupled with information known in the art at the time the patent application was filed. *U.S. v. Telectronics Inc.*, 857 F.2d 778, 8 U.S.P.Q.2d 1217 (Fed. Cir. 1988). Subject matter that is well known to the skilled artisan is preferably omitted from the specification. *Hybritech Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384 (Fed. Cir. 1986) ("a patent need not teach, and preferably omits, what is well known in the art"). Moreover, one skilled in the art is presumed to use the available information in attempting to make and use the claimed invention. *See Northern Telecom, Inc. v. Datapoint Corp.*, 908 F.2d 931, 941 (Fed. Cir. 1990) ("A decision on the issue of enablement requires determination of whether a person skilled in the pertinent art, using the knowledge available to such a person and the disclosure in the patent document, could make and use the invention without undue experimentation."). These enablement rules preclude the need for the Applicant to "set forth every minute detail regarding the invention." *Phillips Petroleum Co. v. United States Steel Corp.*, 673 F. Supp. 1278, 1291 (D. Del. 1991); *see also, DeGeorge v. Bernier*, 768 F.2d 1318, 1323 (Fed. Cir. 1985).

A disclosure adequately fulfills the enablement requirement if it defines the desired functional relationship, even if some experimentation is required. *Wilden Pump & Eng'r Co. v. Pressed & Welded Prod. Co.*, 199 U.S.P.Q. 390 (N.D. Cal. 1978), *aff'd,* 655 F.2d 984, 213 U.S.P.Q. 282 (9th Cir. 1981), *on remand*, 570 F.Supp. 224, 224 U.S.P.Q. 1074 (N.D. Cal. 1983) ("A patent's disclosure is adequate if it defines the desired functional relationship, even if some experimentation is required to reproduce the invention."). *See also, S.C. Johnson & Son, Inc. v. Carter-Wallace, Inc.*, 225 U.S.P.Q. 1022 (S.D.N.Y. 1985); *aff'd in part, vacated in part, and remanded,* 781 F.2d 198, 228 U.S.P.Q. 367 (Fed. Cir. 1986), *on remand,* 231 U.S.P.Q. 668 (S.D.N.Y. 1986) ("There is no need for a manufacturing specification. There need not be a description of every nut, bolt and detail used in the practice of the invention."); *In re Certain Limited-Charge Cell Culture Microcarriers*, 221 U.S.P.Q. 1165, 1174 (U.S. Int'l Trade Comm. 1983), *aff'd sub nom., Massachusetts Institute of Technology v. AB*

Fortia, 774 F.2d 1104, 227 U.S.P.Q. 428 (Fed. Cir. 1985) ("[T]he fact that experimentation may be complex . . . does not necessarily make it undue, if the art typically engages in such experimentation.").

Undue experimentation is experimentation that would require a level of ingenuity beyond what is expected from one of ordinary skill in the field. *Fields v. Conover,* 170 U.S.P.Q. 276, 279 (C.C.P.A. 1971). The factors that can be considered in determining whether an amount of experimentation is undue have been listed in *In re Wands,* 858 F.2d 731, 8 U.S.P.Q.2d 1400, 1404 (Fed. Cir. 1988). Among these factors are: the guidance provided by the specification, the presence of working examples, the amount of pertinent literature, and the level of skill in the art. The test for undue experimentation is not merely quantitative, however, since a considerable amount of experimentation is permissible, so long as it is merely routine. *Id.*

While the predictability of the art can be considered in determining whether an amount of experimentation is undue, mere unpredictability of the result of an experiment is not a consideration. Indeed, the Court of Customs and Patent Appeals has specifically cautioned that the unpredictability of the result of an experiment is not a basis to conclude that the amount of experimentation is undue. *In re Angstadt,* 190 U.S.P.Q. 214, 219 (C.C.P.A. 1976). Further with respect to unpredictability, the court has specifically stated that

> Appellants have apparently not disclosed *every* catalyst which will work; they have apparently not disclosed *every* catalyst which will not work. The question, then, is whether in an unpredictable art, section 112 requires disclosure of a test with *every* species covered by a claim.
>
> * * *
>
> [S]uch a requirement would force an inventor seeking adequate patent protection to carry out a prohibitive number of actual experiments. This would tend to discourage inventors from filing patent applications in an unpredictable area . . . .

Id. (emphasis in original). The *Angstadt* court went on to hold that applicants had indeed

enabled their method for catalytically oxidizing hydrocarbons, stating that

> the proposition that the disclosure must provide "guidance which will enable one skilled in the art to determine, *with reasonable certainty before performing the reaction*, whether the claimed product will be obtained" . . . is contrary to the basic policy of the Patent Act, which is to encourage disclosure of inventions and thereby to promote progress in the useful arts.
>
> * * *
>
> Depriving inventors of claims which adequately protect them and limiting them to claims which practically invite appropriation of the invention while avoiding infringement inevitably has the effect of suppressing disclosure.

Id., *quoting and criticizing In re Rainer*, 54 C.C.P.A. 1445, 377 F.2d 1006, 153 U.S.P.Q. 802 (1967) (emphasis in original).

With respect to the enablement requirement and claim scope, it is well-settled that the inclusion of undisclosed species within a broad genus does not necessarily render a claim unduly broad. *Horton v. Stevens*, 7 U.S.P.Q.2d 1245, 1247 (Bd. Pat. App. & Int'f 1988) ("The mere fact that a claim embraces undisclosed or inoperative species or embodiments does not necessarily render it unduly broad."). Inoperative species within a broad claim are clearly permissible. *Ex parte Cole*, 223 U.S.P.Q. 94, 95 (P.T.O. Bd. App. 1983) ("It is always possible to theorize some combination of circumstances which would render a claimed composition or method inoperative, but the art-skilled would assuredly not choose such a combination."); *In re Anderson*, 471 F.2d 1237, 1242, 176 U.S.P.Q. 331 (C.C.P.A. 1973) ("It is always possible to put something into a combination to render it inoperative. It is not the function of claims to *exclude* all such matters but to point out what the combination is.") (emphasis in original); *Atlas Powder Co. v. E.I. DuPont De Nemours & Co.*, 750 F.2d 1569, 1576-77, 224 U.S.P.Q. 409, 414 (Fed. Cir. 1984) ("Even if some of the claimed combinations were inoperative, the claims are not necessarily invalid . . . "); *Precision Metal Fabricators Inc. v. Jetstream Systems Co.*, 6 U.S.P.Q.2d 1704, 1709 (N.D. Cal. 1988) ("The enablement requirement does not require that the patent disclose the specific embodiment of the claim; a broad claim can be enabled by the disclosure of a single embodiment.") (emphasis added).

In view of the test for enablement, Applicant respectfully wishes to point out that, contrary to the Examiner's assertion that "[d]iscovery of antisense molecules with 'enhanced specificity' *in vivo* requires further experimentation for which no guidance is taught in the

specification"(July 3, 2001 Office Action at page 6), the specification teaches bcl-2 antisense compounds, synthesis of nuclease-resistant oligonucleotide backbones for bcl-2 antisense compounds, successful entry and localization of bcl-2 antisense compounds into the intended target cell and cellular compartment, use of bcl-2 antisense compounds for inhibiting bcl-2 expression *in vitro*, and methods for administering bcl-2 antisense compounds to a human (*see, e.g.*, page 14, line 16 to page 16, line 34 and Examples 13-16 of the instant specification). Importantly, the nature of antisense technology is that it involves screening of a series of antisense compounds to determine which ones have the desired antisense activity when administered to cells. As such, practitioners of this art are prepared to screen inactive antisense compounds in order to find one that demonstrates the desired antisense activity. As discussed above, enablement is not precluded by the necessity for some experimentation such as routine screening. *Hybritech Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367 (Fed. Cir. 1986).

Moreover, in contrast to the Examiner's contention that "[w]hile the specification teaches cell culture inhibition, no evidence of successful *in vivo* (whole organism) antisense inhibition has been shown, nor do the culture examples correlate with whole organism delivery," the post art convincingly demonstrates that, following the teachings of the instant specification, SEQ ID NO:17 (also known as G3139 or Genasense™) can be administered to humans to treat cancer (*see, e.g.*, Waters et al., 2000, "Phase I clinical and pharmacokinetic study of bcl-2 antisense oligonucleotide therapy in patients with non-Hodgkin's lymphoma", J Clin Oncol. 18:1812-1823; Webb et al., 1997, "BCL-2 antisense therapy in patients with non-Hodgkin lymphoma", Lancet. 349:1137-1141, attached hereto as Exhibits D and E, respectively). Furthermore, SEQ ID NO:17 is currently in clinical trials for the treatment of various human cancers (*see, e.g.*, Delihas, 2001, "Targeting the expression of anti-apoptotic proteins by antisense oligonucleotides", Curr Drug Targets 2:167-180), attached hereto as Exhibit F). Thus, to summarize, the specification teaches bcl-2 antisense oligomers, oligonucleotide modifications for improved stability, penetration into target cells, and antisense-mediated efficacy, while the post art demonstrates that *in vitro* efficacy indeed can correlate with *in vivo* efficacy. In particular, as performed by Applicant with *in vitro* experiments, antisense oligomers have been administered to humans as a simple saline solution to successfully treat human cancer (*see, e.g.*, Waters et al., 2000, "Phase I clinical and pharmacokinetic study of bcl-2 antisense oligonucleotide therapy in patients with

non-Hodgkin's lymphoma", J Clin Oncol. 18:1812-1823; Webb et al., 1997, "BCL-2 antisense therapy in patients with non-Hodgkin lymphoma", Lancet. 349:1137-1141).

The Examiner has acknowledged "isolated successes" with antisense therapy *in vivo*. Using the compositions and methods taught in the specification, the Applicant has followed the teachings of the specification to contribute to those successes *in vivo*. In particular, the teachings of the original specification with respect to the target DNA sequence, methods of making and using antisense compounds directed to the target DNA sequence, methods of making and using nuclease-resistant antisense compounds for use *in vivo*, and methods of administering such compounds *in vivo*, along with the state of the art of the time of filing and the post art successes (accomplished by following the teachings of the specification), together clearly prove enablement. As such, Applicant respectfully submits that the original specification provides adequate guidance for the skilled artisan to make and use the claimed invention. As such, Applicant respectfully submits that the rejections of claims 8-24 under 35 U.S.C. §112, first paragraph have been obviated and overcome, and therefore Applicant respectfully requests that the rejections under 35 U.S.C. §112, first paragraph be withdrawn.

CONCLUSION

Applicant respectfully requests entry of the foregoing amendments and remarks into the file of the above-identified application. Applicant believes that each ground for rejection or objection has been overcome or obviated, and that all of the pending claims are in condition for allowance. Withdrawal of all outstanding rejections and objections is therefore respectfully requested. An early allowance is earnestly sought.

Respectfully submitted,

by: Jacqueline Zinn
Reg No. 43,492

Laura A. Coruzzi 30,742

Laura A. Coruzzi (Reg. No.)

Date: November 5, 2001

PENNIE & EDMONDS LLP
1155 Avenue of the Americas
New York, New York 10036-2711
(212) 790-9090

Enclosures

EXHIBIT A

Marked up version of replacement paragraphs

On page 1, line 1, replace the paragraph beginning "This is a" with the following paragraph:

This is a continuation application of U.S. [Serial] Application No. 09/375,514, filed August 17, 1999, which is a continuation application of U.S. Application No. 09/080,285, filed May 18, 1998 and issued as U.S. Patent No. 6,040,181 on March 21, 2000, which is a continuation of Application No. 08/465,485, filed June 5, 1995 and issued as U.S. Patent No. 5,831,066 on November 3, 1998, which is a continuation of Application No. 08/124,256 filed September 20, 1993 (abandoned), which is a continuation-in-part of Application No. 07/840,716, filed February 21, 1992 (abandoned), which is a continuation-in-part of Application No. 07/288,692, filed December 22, 1988 (abandoned).

On page 8, line 4, replace the paragraph beginning "Figure 13" with the following paragraph:

Figure 13 shows optimization of antisense bcl-2 oligomer sequences using the oligonucleotides 5'-TCTCCCAGCGTGCGCCAT-3' (SEQ ID NO:17), 5'-TGCACTCACGCTCGGCCT-3' (SEQ ID NO:18), 5'-GCGCGGCGGGCGGGCGGGCA-3' (SEQ ID NO:26), 5'-GGGCGGAGGCCGGCCGGCGG-3' (SEQ ID NO:27), 5'-AGCGGCGGCGGCGGCAGCGC-3' (SEQ ID NO:28) and 5'-GGGCCGGGAAGGGCGCCCGC-3' (SEQ ID NO:29).

EXHIBIT B

Marked up version of amended claims

8. (amended) [The] A method of treating a bcl-2 related disorder in a human comprising administering an [effective] amount of an anticode oligomer effective for treating said bcl-2 related disorder, wherein said anticode oligomer is from 10-40 bases in length, and wherein said anticode oligomer hybridizes to the nucleic acid sequence of SEQ ID [NO. 19.] NO:19.

9. (amended) A method of treating cancer in a human comprising administering an [effective] amount of an anticode oligomer effective for treating said cancer, wherein said anticode oligomer is from 10-40 bases in length, and wherein said anticode oligomer hybridizes to the nucleic acid sequence of SEQ ID [NO. 19.] NO:19.

10. (amended) The method of Claim [8 or] 9, [wherein said] further comprising administering one or more chemotherapeutic agents [are administered in combination with said anticode oligomer].

11. (amended) The method of Claim [8 or 9] 10 or 25, wherein [said combination] the administration of said anticode oligomer and said one or more chemotherapeutic agents increases the sensitivity of said [disorders to] disorder or cancer said one or more chemotherapeutic agents.

12. (amended) The method of Claim [8 or] 9, wherein said disorder is [selected from the group comprising] non-Hodgkin's lymphoma, prostate cancer, breast cancer, gastro-intestinal cancer or colon cancer.

14. (amended) A pharmaceutical composition comprising an amount of [the] an anticode oligomer [of any of Claims 1-7] effective to prevent or inhibit a bcl-2 related disorder

in a human, wherein said anticode oligomer is from 10-40 bases in length, and wherein said anticode oligomer hybridizes to the nucleic acid sequence of SEQ ID NO:19 [; and a pharmaceutically acceptable carrier].

15. (amended) A method for increasing the sensitivity of a tumor [cells] cell to a chemotherapeutic [agents,] agent, comprising administering to [the tumor cells] said cell an amount of an anticode oligomer effective for increasing the sensitivity of said cell to said chemotherapeutic agent; wherein said cell expresses the human bcl-2 gene; wherein said anticode oligomer is from 10-40 bases in length, and wherein said anticode oligomer hybridizes to the nucleic acid sequence of SEQ ID [NO. 19.] NO:19.

17. (amended) A method of killing a tumor [cells] cell [wherein said cells express] that expresses the human bcl-2 gene, comprising administering to [the tumor cells] said cell an amount of one or more chemotherapeutic agents and [an] anticode [oligomer] oligomers effective for killing said cell, wherein said anticode oligomer is from 10-40 bases in length, and wherein said anticode oligomer hybridizes to the nucleic acid sequence of SEQ ID NO. [NO. 19.] NO:19.

19. (amended) The method [as in any of Claims 15 to 18] of Claim 8, 9, 14, 15 or 17, wherein said anticode oligomer hybridizes to the [nucleic acid sequence TCTCCCAGCGTGCGCCAT (SEQ ID NO. 17)] first six codons of the human bcl-2 open reading frame.

20. (amended) The method [as in any Claims] of Claim 10, 15 [, to] or 25, wherein said chemotherapeutic agent comprises DTIC (decarbazine), Ara-C (cytosine arabinoside), MTX (methotrexate), taxol, cisplatin, etoposide, mitozantron, 2-chlorodeoxyadenosine, dexamethasone, mAMSA, hexamethyl melamine, mitroxantrone, [antimetabolites] an antimetabolite, [alkylating agents] an alkylating agent, [plant alkaloids] a plant alkaloid, [antibiotics] an antibiotic, [and derivatives] or a derivative thereof.

21. (amended) The method of Claim 20 wherein said antimetabolite comprises

methotrexate, 5-fluorouracil, 6-mercaptopurine, cytosine arabinoside, hydroxyurea, [and] or 2-chlorodeoxy adenosine.

22. (amended) The method of Claim 20 wherein said alkylating agent comprises cyclophosphamide, melphalan, busulfan, cisplatin, paraplatin, chlorambucil, [and] or a nitrogen [mustards] mustard.

23. (amended) The method of Claim 20 wherein said plant alkyloid comprises vincristine, vinblastine, [and] or VP-6.

24. (amended) The method of Claim 20 wherein said antibiotic comprises doxorubicin (adriamycin), daunorubicin, mitomycin c, [and] or bleomycin.

THE LANCET

BCL-2 antisense therapy in patients with non-Hodgkin lymphoma

A Webb, D Cunningham, F Cotter, P A Clarke, F di Stefano, P Ross, M Corbo, Z Dziewanowska

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Early report

BCL-2 antisense therapy in patients with non-Hodgkin lymphoma

A Webb, D Cunningham, F Cotter, P A Clarke, F di Stefano, P Ross, M Corbo, Z Dziewanowska

Summary

Background Overexpression of BCL-2 is common in non-Hodgkin lymphoma and leads to resistance to programmed cell death (apoptosis) and promotes tumorigenesis. Antisense oligonucleotides targeted at the open reading frame of the *BCL-2* mRNA cause a specific down-regulation of BCL-2 expression which leads to increased apoptosis. Lymphoma grown in laboratory animals responds to *BCL-2* antisense oligonucleotides with few toxic effects. We report the first study of *BCL-2* antisense therapy in human beings.

Methods A daily subcutaneous infusion of 18-base, fully phosporothioated antisense oligonucleotide was administered for 2 weeks to nine patients who had BCL-2-positive relapsed non-Hodgkin lymphoma. Toxicity was scored by the common toxicity criteria, and tumour response was assessed by computed tomography scan. Efficacy was also assessed by quantification of BCL-2 expression; BCL-2 protein levels were measured by flow cytometry in samples from patients.

Findings During the course of the study, the daily dose of *BCL-2* antisense was increased incrementally from 4·6 mg/m² to 73·6 mg/m². No treatment-related toxic effects occurred, apart from local inflammation at the infusion site. In two patients, computed tomography scans showed a reduction in tumour size (one minor, one complete response). In two patients, the number of circulating lymphoma cells decreased during treatment. In four patients, serum concentrations of lactate dehydrogenase fell, and in two of these patients symptoms improved. We were able to measure BCL-2 levels by flow cytometry in the samples of five patients, two of whom had reduced levels of BCL-2 protein.

Interpretation In patients with relapsing non-Hodgkin lymphoma, *BCL-2* antisense therapy led to an improvement in symptoms, objective biochemical and radiological evidence of tumour response, and down-regulation of the BCL-2 protein in some patients. Our findings are encouraging and warrant further investigations of *BCL-2* antisense therapy in cancer treatment.

Lancet 1997; 349: 1137–41

Lymphoma Unit, Royal Marsden Hospital, Sutton, Surrey SM2 5PT, UK (A Webb MRCP, D Cunningham FRCP); **CRC Centre for Cancer Therapeutics, Institute of Cancer Research, Sutton** (P A Clarke PHD, F di Stefano MSC, P Ross MRCP); **Institute of Child Health, London** (F Cotter FRCP, M Corbo MSC); **and Genta Incorporated, San Diego, California, USA** (Z Dziewanowska MD)

Correspondence to: Dr D Cunningham

Introduction

Advanced stage, low-grade, follicular non-Hodgkin lymphomas are incurable. However, such tumours are indolent and chemosensitive, and after a diagnosis has been made, a patient's median survival is about 10 years. In the later stages of the disease, these tumours develop chemoresistance and often a more aggressive histological appearance. Intermediate or high-grade non-Hodgkin lymphomas also respond well to combination chemotherapy, such as cyclophosphamide, doxorubicin, vincristine, prednisolone, with or without radiotherapy, but only about 40% of patients become long-term survivors. The use of new drugs, different chemotherapy combinations, and high-dose chemotherapy with bone-marrow or peripheral stem-cell rescue have had an impact, but the effect on the number of long-term survivors has been limited. An alternative approach is treatment based on the molecular biology of the disease, which may improve response and survival.

Overexpression of the BCL-2 gene results in resistance to programmed cell death (apoptosis),[1] which leads to chemoresistance.[2] Two studies of the prognostic significance of BCL-2 overexpression in patients with diffuse, large B-cell lymphoma, confirmed the importance of BCL-2 expression as an independent prognostic marker.[3,4] However, in low-grade, follicular non-Hodgkin lymphoma, determination of BCL-2 overexpression is of no value because most tumours overexpress BCL-2. High concentrations of BCL-2 protein expression occur for several reasons, one of these being a t(14;18) translocation. However, the prognostic significance of this specific translocation in low-grade and more aggressive lymphomas has not been shown.[4,5]

Antisense oligonucleotides are short, single-stranded DNA molecules (15–25 bases long) that are complementary to a key region of the mRNA of the target gene and are able to reduce gene expression. There is strong in-vitro evidence that fully phosphorothioated antisense oligonucleotides complementary to the open reading frame of the *BCL-2* mRNA can down-regulate BCL-2 expression, which results in reduced cell viability.[6] Cotter and colleagues[7] used a severe combined immunodeficient mouse lymphoma model to show that a subcutaneous infusion of an 18-base, fully phosphorothioated oligonucleotide for 2 weeks had a potent antitumour effect. This in-vitro and in-vivo data enabled us to begin a phase I trial in patients with relapsing lymphoma and high BCL-2 expression. We report here our preliminary findings.

Methods

Eligible patients were men or women who had non-Hodgkin lymphoma of any histological grade with immunohistochemical evidence of overexpression of the BCL-2 protein on lymph-node biopsy samples. In addition, patients had to have relapsing disease after the completion of at least two chemotherapy regimens, a life expectancy of more than 12 weeks, normal renal and liver

Patient number	Age	Sex	Dose of BCL-2 antisense (mg/m²)	Histological classification		Number of previous chemotherapy regimens	Bone marrow	Circulating lymphoma cells	Palpable peripheral lymphadenopathy
				Working formulation	REAL				
1	50	M	4·6	B	Follicular grade 1	2	–	–	–
2	63	M	9·2	E	Mantle cell	3	+	+	+
3	64	M	18·4	G	Diffuse large B cell	4	–	–	–
4	68	M	36·8	B	Follicular grade 1	5	–	–	+
5	41	F	36·8	B	Follicular grade 1	2	–	–	–
6	65	F	36·8	B	Follicular grade 1	5	+	+	+
7	67	F	73·6	A	Small lymphocytic	4	+	+	+
8	53	M	73·6	C	Follicular grade 2	4	–	–	–
9	54	F	73·6	G	Diffuse large B cell	4	–	–	–

REAL=Revised European American Lymphoma classification.

Table 1: Baseline characteristics of patients

function, a white-blood-cell count of more than 3×10^9/L, and a platelet count of more than 100×10^9/L.

A daily dose of an 18-base, fully phosphorothioated oligonucleotide (sequence 5'-TCTCCCAGCGTGCGCCAT-3, dissolved in isotonic normal saline) was administered as a subcutaneous continuous infusion with a portable syringe driver. This oligonucleotide was complementary to the first six codons of mRNA of the *BCL-2* gene. Infusion sites were changed when we observed early signs of inflammation. Treatment-related toxicity was scored by the common toxicity criteria;[8] we included areas of concern pinpointed in animal and in-vitro studies.[7] Toxicity was monitored for the first 48 h of treatment while the patients were in hospital, but thereafter on an outpatient basis. One 2-week course of treatment was given. Patients were followed up for 4 weeks after the end of treatment. If there was evidence of tumour response, a second course was considered. The initial daily dose was 4·6 mg/m², which was equivalent to one tenth of the dose that would kill 10% of mice (LD_{10}). This dose was then increased by 100%, unless grade 2 or higher toxicity was observed according to the European Organization for Research and Treatment of Cancer criteria.[9] We defined the maximum tolerated dose as that which caused grade 3 or 4 toxicity in at least 50% of patients.

Tumour response was assessed by computed tomography scanning before the start of treatment, at week 2 (end of infusion), and at week 6. We used WHO[10] criteria for the classification of tumour response. A complete response was defined as disappearance of all disease. A partial response was a 50% or more reduction in the bidimensional product of measurable disease. An increase in the bidimensional product of measurable disease of more than 25% was defined as progressive disease. All other states were defined as stable disease. Additional indices of lymphoma activity were serum concentrations of lactate dehydrogenase and the number of circulating lymphoma cells identified morphologically (changes of more than 20% were deemed relevant).

Samples of blood, bone marrow, and fine-needle aspirates of lymph nodes were collected at weeks 0 (start of treatment), 2, and 6. Mononuclear cells, freshly separated by Ficoll-Isopaque centrifugation, were suspended in 10% dimethyl sulphoxide and stored in liquid nitrogen. At the time of analysis, the samples were fixed in 70% ethanol, incubated with an antibody to the BCL-2 protein (DAKO, clone 124) followed by further incubation with anti-immunoglobulin G labelled with fluorescein isothiocyanate. Levels of BCL-2 protein were measured by flow cytometry of gated lymphocytes. Within this population of cells, those positive and negative for BCL-2 were identified. The mean (SD) levels of BCL-2 protein were calculated by gating on those positive for BCL-2. All samples from each patient were labelled simultaneously under the same conditions. Because changes in the concentration of BCL-2 could reflect general changes in all protein expression, we used non-specific changes in another protein (from patient 3 onwards) as the control; these levels were measured by flow cytometry and samples were incubated with fluorescein-conjugated HLA-A, B, C antibody. The levels of HLA were consistent between individual samples for each patient.

	Common toxicity criteria grade			
	0	I	II	III
Haematological toxicity				
Anaemia	6	..	3*	..
Leucopenia	8	–	–	1†
Lymphopenia	5	2	–	2*
Thrombocytopenia	7	–	2*†	–
Clotting	9	–	–	–
Non-haematological toxicity				
Renal	9	–	–	–
Neurological	9	–	–	–
Gastrointestinal	9	–	–	–
Liver	9	–	–	–
Pulmonary	9	–	–	–
Cardiovascular	8	–	–	1‡
Hyperglycaemia§	0	5	4	–
Infection‖	5	1	2	1
Local skin reaction	0	6	3	–

*Progressive lymphomatous disease. †Secondary to septicaemia. ‡Secondary to obstruction of superior vena cava. §Without fasting. ‖Coincidental secondary to advanced lymphoma.

Table 2: Toxic effects of treatment

Results

All patients had overexpression of BCL-2 protein on immunohistochemical staining of lymphoma cells from biopsy specimens before treatment. During a 6-month period, nine eligible patients were treated. Six patients had low-grade tumour and three had intermediate or high-grade tumour histology. All patients had stage IV non-Hodgkin lymphoma (table 1).

Toxic effects of treatment

Dose escalation at 100% increments was possible, as planned, due to low toxicity. There was no antisense-related haematological toxicity (table 2). However, patient 8 developed grade 3 leucopenia and grade 2 thrombocytopenia associated with a *Haemophilus influenzae* chest infection at the start of treatment. Antisense treatment was continued in patient 8, and

Patient number	Response on computed tomography	Circulating lymphoma cells	Serum lactate dehydrogenase	B symptoms	BCL-2 protein levels
1	SD	NE	↓	↓	NE
2	SD	↓	↓	→	PB↓,BM↓,LN→
3	PD	NE	↑	→	NE
4	PD	NE	↑	→	LN→
5	PD	NE	→	→	BM→
6	SD*	↓	↓	→	LN↓,BM→,PB→
7	PD	→	↑	→	LN→,BM→,PB-NE
8	CR	NE	↓	↓	NE
9	PD	NE	→	→	NE

CR=complete response; SD=stable disease; PD=progressive disease; NE=non-evaluable; LN=lymph node; BM=bone marrow; PB=peripheral blood sample.
*Tumour shrinkage but did not satisfy definition of partial response.

Table 3: Tumour response

Figure 1: **Computed tomography scan for patient 8**
A: pretreatment; B: at 6 weeks after start of treatment, showing complete resolution of left axillary lymphadenopathy.

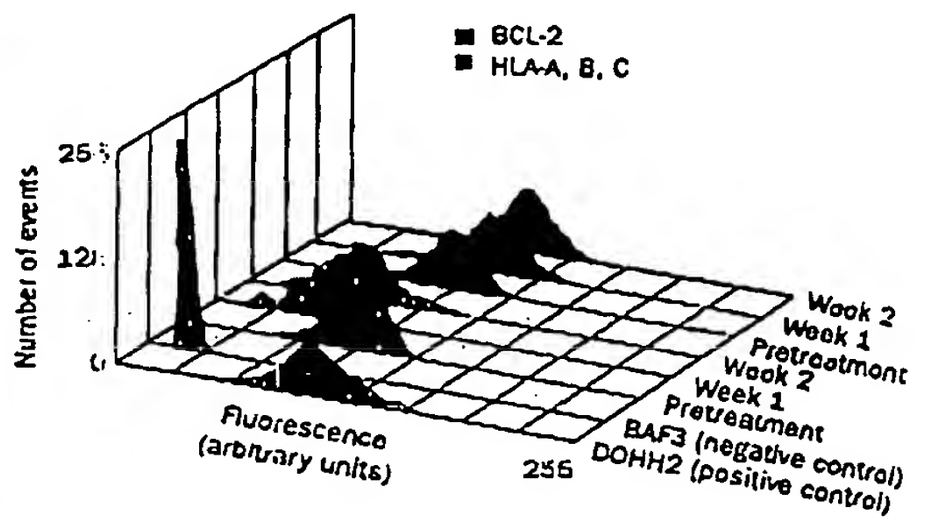

Figure 2: **Histograms of BCL-2 levels measured by flow cytometry in lymph node fine-needle aspirates of patient 6 during 2-week treatment period**
BCL-2 levels are reduced at weeks 1 and 2 compared with pretreatment, whereas HLA-A, B, C levels (control protein) do not change. BAF3 cell line and DOHH2 cell line act as negative and positive controls, respectively.

leucopenia and thrombocytopenia resolved after treatment with intravenous antibiotics, which suggested a non-oligonucleotide effect. Patient 9 developed grade 2 thrombocytopenia at the end of the 2nd week, and moderate (up to 31%) eosinophilia was observed. Subsequently, at week 6, infiltration of bone marrow and progressive disease in lymph nodes was observed. Because the thrombocytopenia and eosinophilia resolved with subsequent chemotherapy, these effects were more likely to result from advanced-stage lymphoma than from the antisense oligonucleotide. Lymphopenia was present in four patients (patients 3, 7, 8, and 9) at the start of treatment and did not worsen during antisense therapy. Anaemia was observed in three patients (patients 2, 5, and 9), but was not dose related and seemed to be associated with advanced infiltration of bone marrow. No clotting abnormalities (prothrombin or partial thromboplastin times or fibrinogen) or treatment-related changes in the CD4/CD8 ratio were observed. Repeated samples of bone marrow aspirates and trephines showed no evidence of treatment-related aplasia.

Non-haematological toxic effects are shown in table 2. Patient 7 had episodes of transient syncope during rest 2 days after the end of treatment. These episodes were caused by obstruction of the superior vena cava due to progressive mediastinal disease. After chemotherapy to reduce this obstruction, no further episodes have occurred. All nine patients had a transient increase in non-fasting blood concentrations of glucose, but none exceeded 12 mmol/L and all patients' blood glucose concentrations returned to within the normal range after stopping antisense therapy. No intervention was required and the degree of hyperglycaemia was not dose related. Four patients developed an infection but no infection was directly attributable to the antisense therapy. Antisense treatment had no effect on liver function. The only significant toxic effect of antisense therapy was a local skin reaction around the infusion site. In eight patients this reaction simply required resiting of the line every 3–4 days. However, one patient (patient 4) had a local inflammatory reaction that became unacceptably painful about 12 h after the start of treatment. A skin biopsy sample from the inflamed area showed perivascular and periadnexal infiltrate in the dermis, consisting of T lymphocytes (unable to identify subtypes), histiocytes, and a few plasma cells. The epidermis was normal and there was no vasculitis. Despite several site changes and reduction of drug concentration by 50%, the inflammation persisted and treatment was stopped. Two patients who received the same dose and three who received a 100% dose increment did not have the same degree of reaction as patient 4.

Tumour response

The response to treatment is shown in table 3. At week 6, patient 8 had no lymph-node masses larger than 1 cm and had achieved a response in all lymph-node sites; the largest pretreatment lymph node measured 2·5×2·0 cm (figure 1). This patient remained in remission 4 months after the start of treatment without any further therapy. At week 6, patient 6 had near partial responses in right axillary and mediastinal lymph nodes, but in the larger lymph-node mass within the abdomen, response was negligible. Two patients (patients 1 and 8) had improvements in their B symptoms. Patient 1 had resolution of sweats and pruritus within 48 h of the start of treatment, which lasted for 3 weeks. During a second course of treatment at the same dose, this patient again had resolution of sweats and pruritus. In patient 8, lymphoma-related alcohol intolerance resolved so that he was able to drink alcohol for the first time in 2 years. Two of three patients (patients 2 and 6) had a reduction in their numbers of circulating lymphoma cells. Serum concentrations of lactate dehydrogenase decreased in four patients (patients 1, 2, 6, 8).

BCL-2 expression

Levels of BCL-2 protein were measured by flow cytometry in peripheral blood samples containing circulating lymphoma cells in three patients (patients 2, 6, 7), in lymphoma-infiltrated bone-marrow aspirates in four patients (patients 2, 5, 6, 7), and in fine-needle aspirates of peripheral lymph nodes in three patients (patients 2, 4, 6, 7). We were not able to measure BCL-2 levels in patients who had impalpable lymph nodes (patients 1, 3, 5, 8, 9) or no lymphoma cells in blood or bone marrow. The levels of HLA-A, B, C (control protein) did not differ significantly between individual patient's samples. The levels of BCL-2 protein decreased in the peripheral blood and bone marrow aspirates of patient 2 and in the lymph node aspirates of patient 6 (figure 2). BCL-2 protein concentrations in the samples that contained lymphoma cells from patients 6 and 7 showed little change.

Further treatment

Eight patients have gone on to receive further chemotherapy with various regimens, six of whom have achieved a partial response. Patient 9 was treated with a chemotherapy regimen of chlorambucil, vinblastine, procarbazine, and prednisolone (ChlVPP) 5 weeks after the end of antisense treatment and developed complete alopecia.

Discussion

In-vitro and in-vivo studies of antisense oligonucleotides to inhibit expression of BCL-2 have shown good efficacy with low toxicity.[7] Our study shows that *BCL-2* antisense therapy leads to tumour regression, improvement in the biochemical and haematological variables and symptoms, and down-regulation of BCL-2 expression. Our findings are encouraging because the maximum tolerated dose has not been reached; the doses used were just above those that are effective in the mouse model. Two patients had evidence of tumour shrinkage on computed tomography scanning and one patient maintained this improvement 4 months after treatment. Both patients had low-grade tumours. In rare cases, low-grade lymphomas can regress spontaneously, although it is unlikely that such regression would occur in these two patients by chance within a few weeks of antisense therapy. Furthermore, both patients had decreased numbers of circulating lymphoma cells and concentrations of serum lactate dehydrogenase. In addition, two patients had relief of symptoms. Together, these findings indicate that *BCL-2* antisense oligonucleotides have antilymphoma activity. Cotter and colleagues' in-vitro study[8] of *BCL-2* antisense reported specific reduction in mRNA, protein expression, and induction of apoptosis. Our findings are the first reported evidence of down-regulation of BCL-2 protein in human beings.

An important aim of this study was to establish the toxicity of *BCL-2* antisense oligonucleotides. At the dose reached to date, we have found no substantial dose-limiting toxic effects. Therefore, the dosage in future patients will be increased to determine the maximum tolerated dose. Concerns about potential toxicity of *BCL-2* antisense oligonucleotides come from various sources. Data on toxicity in mice showed myocardial and liver necrosis, splenomegaly, and deaths at high daily dosages (50 mg/kg). The doses used in our study are substantially below those at which toxicities were observed in mice. Primate studies showed no clinical toxicity and necropsy revealed only mild inflammation at the injection site and non-specific pathological changes in the kidneys at daily doses of up to 10 mg/kg for 2 weeks.[7] Galbraith and colleagues' study[11] of high doses of other phosphorothioate compounds in monkeys reported hypotension and death if the drugs were given as a rapid intravenous bolus but not when they were given as an infusion. Furthermore, the hypotensive events were not sequence related.[11] Bishop and colleagues' study[12] of daily doses of up to 6 mg/kg of an intravenous infusion of a 20-base fully phosphorothioated oligonucleotide targeted against *p53* in patients with acute myeloid leukaemia and myelodysplastic syndrome showed no major drug-related toxicity.[12]

There was concern that *BCL-2* antisense therapy might affect cells that express high concentrations of BCL-2 protein under normal conditions—for example, memory B cells, mature T cells (CD4 and CD8), neuronal tissue, and intestinal mucosa. Such high concentrations may allow these cells to have long life spans. Bone-marrow stem cells do not usually overexpress BCL-2 and another member of the *BCL-2* family, *BCL-X*$_L$, seems to be of greater importance here, so adverse effects against stem cells are unlikely.[13] Furthermore, it seems unlikely that *BCL-2* antisense therapy would damage neuronal tissue because radiolabelled *BCL-2* antisense oligonucleotides were not detectable in the brain of rodents.[14] In addition, mice in whom both copies of the *BCL-2* gene have been deleted by recombinant DNA techniques (knockout mice) are viable without neurological deficit, which suggests that *BCL-2* is not of prime importance to the stem cells or neuronal tissue. However, the knockout mice do subsequently develop lymphopenia, apoptotic involution in the thymus and spleen, polycystic kidneys, grey hair in the second follicle cycle,[15,16] and distorted small intestine.[16]

The only significant toxic effect of *BCL-2* antisense therapy was an inflammatory response at the injection site. In one patient this response was severe enough for the treatment to be stopped. However, response of this severity did not occur in the other patients, even at higher doses. We also noted transient non-fasting hyperglycaemia during the treatment period. The effect had no clinical implications, but has already been reported by Bishop and colleagues,[12] which suggests that it may be associated with the phosphorothioate backbone chemistry and not with the dose. These investigators also reported a transient increase in concentrations of liver aminotransferases in two patients, which resolved after the completion of treatment.[12] Another trial (targeting HIV) of phosphorothioates given over 2 h noted a transient increase in partial thromboplastin times not associated with clotting deficiency.[17] By contrast, we did not find any changes in clotting factors or in concentrations of liver aminotransferases.

BCL-2 antisense oligonucleotides could potentially be used as a single agent in the treatment of minimal residual disease or ex-vivo purging of bone marrow or peripheral stem-cell harvests. Perhaps the most important potential application of *BCL-2* antisense therapy may be to overcome chemoresistance. We found that six of eight patients who were treated with chemotherapy after antisense treatment went on to achieve a partial remission. In addition, patient 9 developed complete alopecia with a chemotherapy regimen that has a documented 0·5% incidence of this side-effect.[18] BCL-2 levels in the hair follicle are high,[19] and *BCL-2* knockout mice develop

Phase I Clinical and Pharmacokinetic Study of Bcl-2 Antisense Oligonucleotide Therapy in Patients With Non-Hodgkin's Lymphoma

By Justin S. Waters, Andrew Webb, David Cunningham, Paul A. Clarke, Florence Raynaud, Francesca di Stefano, and Finbarr E. Cotter

Purpose: To evaluate the pharmacokinetics and toxicity of an antisense oligonucleotide targeting *bcl-2* in patients with non-Hodgkin's lymphoma (NHL) and to determine efficacy using clinical and biologic end points.

Patients and Methods: Twenty-one patients with Bcl-2–positive relapsed NHL received a 14-day subcutaneous infusion of G3139, an 18-mer phosphorothioate oligonucleotide complementary to the first six codons of the *bcl-2* open reading frame. Plasma pharmacokinetics were measured by anion exchange high-performance liquid chromatography. Response was assessed by computed tomography. Changes in Bcl-2 expression were measured by fluorescence-activated cell sorting of patients' tumor samples.

Results: Eight cohorts of patients received doses between 4.6 and 195.8 mg/m^2/d. No significant systemic toxicity was seen at doses up to 110.4 mg/m^2/d. All patients displayed skin inflammation at the subcutaneous infusion site. Dose-limiting toxicities were thrombocytopenia, hypotension, fever, and asthenia. The maximum-tolerated dose was 147.2 mg/m^2/d. Plasma levels of G3139 equivalent to the efficacious plasma concentration in in vivo models were produced with doses above 36.8 mg/m^2/d. Plasma levels associated with dose-limiting toxicity were greater than 4 μg/mL. By standard criteria, there was one complete response, 2 minor responses, nine cases of stable disease, and nine cases of progressive disease. Bcl-2 protein was reduced in seven of 16 assessable patients. This reduction occurred in tumor cells derived from lymph nodes in two patients and from peripheral blood or bone marrow mononuclear cell populations in the remaining five patients.

Conclusion: Bcl-2 antisense therapy is feasible and shows potential for antitumor activity in NHL. Down-regulation of Bcl-2 protein suggests a specific antisense mechanism.

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ANTISENSE OLIGONUCLEOTIDES are chemically modified single-strand DNA molecules of between 13 and 25 nucleotides in length. They have a nucleotide sequence complementary to that of their target mRNA and are capable of inhibiting expression of the target gene. The specificity of this action at the level of gene expression makes antisense oligonucleotide therapy a powerful tool with wide-ranging potential clinical applications. Clinical trials have been initiated in the fields of malignancy and inflammatory and viral diseases, using oligonucleotides targeting a variety of genes. These trials have demonstrated the feasibility of this therapeutic approach, with some evidence of clinical activity.

The *bcl-2* gene provides a rational target for antisense strategies. Overexpression leads to cellular resistance to programmed cell death (apoptosis),[1] resulting in chemoresistance in vitro.[2] Bcl-2 is overexpressed in the majority of low-grade non-Hodgkin's lymphoma (NHL) cases and approximately 50% of high-grade NHL cases. A number of different molecular mechanisms are responsible for the upregulation of the Bcl-2 protein, the most common being a translocation between chromosomes 14 and 18 that brings the *bcl-2* gene under the transcriptional control of the immunoglobulin heavy chain promoter. There is evidence for an etiologic role of Bcl-2 overexpression in lymphoma. Transgenic mice with deregulated Bcl-2 expression initially develop lymphoid hyperplasia with extended B-cell survival.[3] In some cases, this progresses to diffuse large B-cell lymphoma, often with the accumulation of additional genetic abnormalities, such as rearrangement of the *c-myc* gene.[4] Three recent studies have examined the prognostic significance of Bcl-2 expression in diffuse large B-cell NHL.[5-7] In multivariate analyses, Bcl-2 expression was found to be an independent poor prognostic factor in these patients.

From the Lymphoma Unit, Royal Marsden Hospital, and Cancer Research Campaign Centre for Cancer Therapeutics, Institute of Cancer Research, Sutton, and Institute of Child Health, London, United Kingdom.

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Address reprint requests to David Cunningham, MD, Department of Medicine, Royal Marsden Hospital, Downs Rd, Sutton, Surrey SM2 5PT, United Kingdom; email dcunn@icr.ac.uk.

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Antisense oligonucleotides targeting *bcl-2* have been demonstrated to reduce *bcl-2* mRNA and protein levels in vitro[8] and to reverse chemoresistance of Bcl-2–expressing lymphoma cell lines.[9] It has also been demonstrated that eradication of lymphoma can be achieved in a severe combined immune deficient mouse model of human NHL by a 14-day subcutaneous infusion of G3139 (Genta Inc, Lexington, MA), an 18-mer phosphorothioate oligonucleotide targeting the first six codons of the *bcl-2* mRNA open reading frame, sequence 5′-tctcccagcgtgcgccat.[10] These observations provided the rationale for a phase I trial of antisense oligonucleotide G3139 in NHL patients.

PATIENTS AND METHODS

Methods for this trial have been described previously.[11] Eligible patients were men or women with B-cell NHL of any histologic subtype, provided there was immunohistochemical evidence of Bcl-2 expression by tumor cells in the biopsy material. Patients were required to have received at least one previous line of chemotherapy and to have progressive disease. Informed consent was obtained from all patients before entry onto the study, which was approved by the Royal Marsden Hospital Ethics Committee. Antisense oligonucleotide G3139 was delivered as a continuous subcutaneous infusion for 14 days by a portable infusion pump. Toxicity was graded according to the common toxicity criteria and assessed during the 2-week treatment period and during the subsequent 4 weeks. One course of treatment was planned per patient, but additional courses of treatment were considered in the event of a tumor response. A second course was administered to patients no. 2, 17, and 21. The initial dose was 4.6 mg/m^2/d, calculated as one tenth of the dose that would kill 10% of mice in preclinical toxicity studies. Planned dose escalation was in 100% increments. One patient was enrolled at each of the first three dose levels, after which three patients per dose level were included. The following were considered dose-limiting toxicities (DLTs): grade 4 leukopenia or neutropenia, grade 3 or 4 thrombocytopenia, any complicated grade 3 or 4 myelosuppression, and any grade 3 or 4 nonhematologic systemic toxicity. In the event of a DLT in one of three patients at a single dose level, the subsequent dose escalation was reduced to 33%. In the event of DLTs in more than one patient at a single dose level, the dose level below was expanded to a maximum of six patients.

Plasma concentrations of G3139 were measured by anion exchange high-performance liquid chromatography, as described previously.[12] Briefly, duplicate standard curves were produced in control plasma at the levels of G3139 0.25, 0.5, 1, 2, 5, 10, and 20 μg/mL. Plasma from standard curves and patient samples was extracted with phenol: chloroform:isoamyl alcohol. Chromatographic separation was achieved on a GenPak-fax column (Waters, Watford, United Kingdom). G3139 eluted with a gradient of LiCl 2 mol/L in LiOH 20 mmol/L. Detection was achieved by spectroscopy at 254 nm. Pharmacokinetics were evaluated by compartmental and noncompartmental analysis using WinNonlin Pro Edition software model 202 (infusion model) (Pharsight, Mountain View, CA). Statistical analysis was performed with Minitab statistical software (Minitab Inc, State College, PA). Relationships between variables were assessed by linear regression and analysis of variance with the general linear model.

Tumor response was assessed primarily by computed tomography (CT) scanning using World Health Organization response criteria, as described previously.[11] Additional parameters of response included tumor-related symptoms, numbers of circulating lymphoma cells in the peripheral blood (assessed morphologically and quantified by an experienced hematologist who counted cells on a blood film or by fluorescence-activated cell sorting [FACS] analysis for CD19 expression), and serum concentrations of lactate dehydrogenase (normalization of a previously elevated level was considered relevant). Bcl-2 protein levels were measured by FACS analysis of mononuclear cells obtained from peripheral-blood and bone marrow aspirates and tumor cells obtained from fine-needle aspirates of palpable lymph nodes collected before, during, and after treatment.[11] To examine the effect of treatment on the target lymphoma cells, this analysis was confined to Bcl-2–expressing cells by gating on the appropriate population.

RESULTS

Pretreatment Characteristics

Twenty-one patients were treated at doses ranging from 4.6 mg/m^2/d to 195.8 mg/m^2/d. Eleven patients were male, and the median age was 54 years (range, 41 to 73 years). The majority had a diagnosis of low-grade NHL (nine had follicular NHL and eight had small lymphocytic NHL), but three patients had diffuse large B-cell lymphoma and one had mantle-cell lymphoma. Patients had received a median of four previous chemotherapy regimens (range, one to eight). Twenty-six different regimens had been used, the most common being chlorambucil (15 patients), cyclophosphamide, doxorubicin, vincristine, and prednisolone (10 patients), and fludarabine (10 patients). Four patients had received high-dose chemotherapy with autologous peripheral stem-cell support, and three patients had received prior radiotherapy (Table 1).

Toxicity

The only hematologic toxicity that seemed to be unequivocally related to G3139 therapy was thrombocytopenia (Table 2). Of the five patients treated at a dose of 147.2 mg/m^2/d, two patients developed grade 2 and one patient developed grade 3 thrombocytopenia, and two patients treated at a dose of 195.8 mg/m^2/d developed grade 3 and grade 4 thrombocytopenia. In all of these cases, the platelet count fell progressively during the course of the G3139 infusion and recovered after discontinuation of treatment. Furthermore, a correlation existed between plasma concentration of G3139 and nadir platelet count (Fig 1), suggesting a dose-dependent effect.

Some hematologic abnormalities observed in this trial seemed to be unrelated to G3139 treatment. Patient no. 8 had grade 3 leukopenia and neutropenia, grade 4 lymphopenia, and grade 2 thrombocytopenia within the first 4 days of therapy. These observations coincided with a *Haemophilus influenzae* pneumonia, and all abnormalities resolved on antibiotic treatment, despite continuation of the G3139 infusion. Grade 2 leukopenia was also observed in patient

Table 1. Patient Characteristics

Patient No.	Sex	Age	Diagnosis (REAL)	No. of Previous Treatments	Dose (mg/m²/d)
1	M	50	Follicular	2	4.6
2	M	63	Mantle cell	3	9.2
3	M	64	Diffuse large B cell	4	18.4
4	M	68	Follicular	5	36.8
5	F	41	Follicular	2	36.8
6	F	65	Follicular	5	36.8
7	F	57	Small lymphocytic	4	73.6
8	M	53	Follicular	4	73.6
9	F	54	Diffuse large B cell	4	73.6
10	M	55	Small lymphocytic	3	147.2
11	M	54	Small lymphocytic	8	147.2
12	F	42	Follicular	4	147.2
13	F	50	Follicular	4	147.2
14	F	73	Small lymphocytic	2	195.8
15	M	51	Diffuse large B cell	5	195.8
16	M	45	Small lymphocytic	7	195.8
17	M	51	Small lymphocytic	7	147.2, 36.8
18	F	51	Small lymphocytic	4	73.6
19	F	44	Follicular	2	110.4
20	M	55	Small lymphocytic	1	110.4
21	F	59	Follicular	1	110.4

Abbreviation: REAL, Revised European and American lymphoma classification system.

no. 13, in the absence of neutropenia; this patient had long-standing lymphopenia that did not worsen during treatment. Grade 3 neutropenia occurred in patient no. 2, but this was a pre-existent abnormality resulting from bone marrow infiltration by lymphoma. Grade 3 or 4 lymphopenia was observed in 10 patients (patients no. 6, 7, 8, 12, 13, 15, 18, 19, 20, and 21) and grade 1 or 2 lymphopenia was seen in a further three patients (patients no. 3, 9, and 10). This predated the start of therapy in most cases but seemed to worsen transiently during treatment in some patients (patients no. 6, 10, 12, 18, 20, and 21), suggesting a possible oligonucleotide-related effect. However, the lymphopenia was not clinically significant. Grade 1 or 2 anemia occurred in 15 of the 21 patients, but this was coincidental with lymphomatous bone marrow infiltration, with no obvious temporal relationship to treatment. No clotting abnormalities or changes in the CD4 to CD8 ratio were observed. A repeat bone marrow examination demonstrated no evidence of hypoplasia or G3139-induced toxicity to bone marrow precursors.

Nineteen patients developed transient grade 1 or 2 nonfasting hyperglycemia during G3139 infusion. There was no

Table 2. Toxicity: Grades 3 and 4

	Dose				
	≤ 36.8 mg/m²/d* (n = 7)	73.6 mg/m²/d (n = 4)	110.4 mg/m²/d (n = 3)	147.2 mg/m²/d* (n = 5)	195.8 mg/m²/d (n = 3)
Leukopenia	0	1	0	0	0
Neutropenia	1	1	0	0	0
Lymphopenia	1	3	3	2	1
Thrombocytopenia	0	0	0	1	2
Anemia	0	0	0	0	0
Renal	0	0	0	0	1
Hypotension	0	0	0	1	0
Fever	0	0	0	1	0
Asthenia	0	0	0	0	1
Skin, local	0	0	0	1	0

NOTE. All grade 3 and 4 events are shown, irrespective of suspected relationship to G3139 therapy. Numbers in parentheses are numbers of patients.
*Patient no. 17 received treatment at 36.8 and 147.2 mg/m²/d.

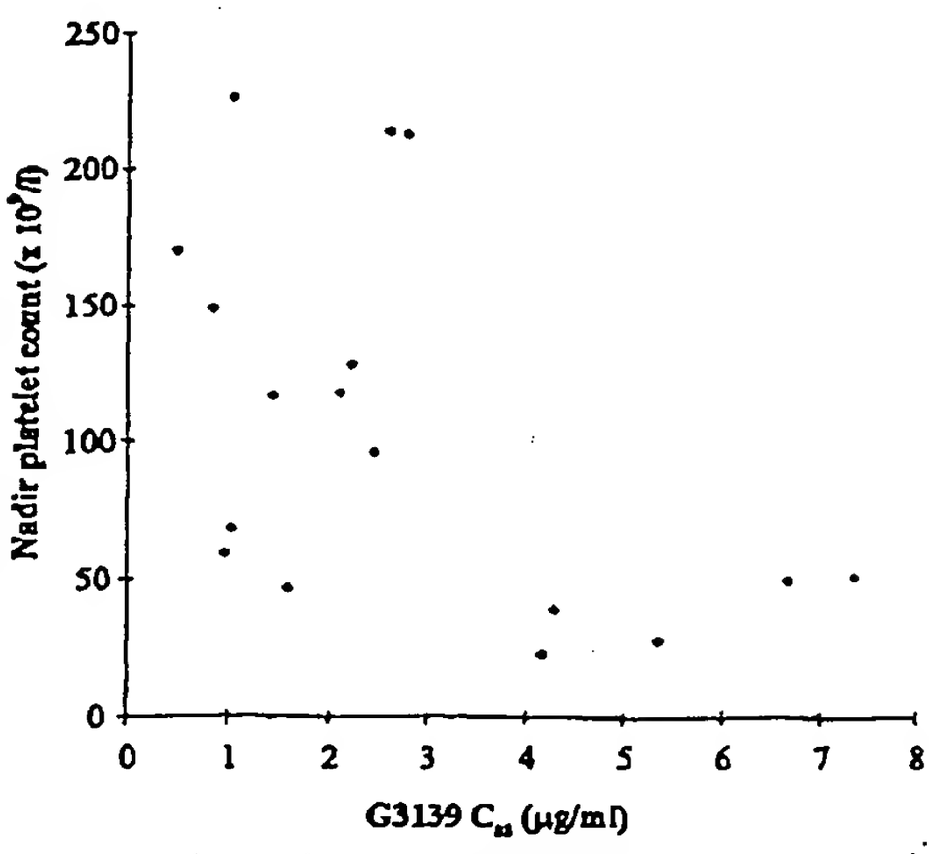

Fig 1. Steady-state plasma concentration of G3139 plotted against nadir platelet count for each patient.

apparent relationship with drug dose. Transient increases in concentrations of hepatic aminotransferases or alkaline phosphatase occurred in seven patients. Slight transient increases in urea and creatinine were also documented in four patients. These abnormalities were more common at higher doses, with the majority occurring in patients treated at 147.2 mg/m^2/d and above. Intervention was not required, and all biochemical abnormalities resolved after completion of the treatment course. All patients experienced local skin inflammation at the sites of subcutaneous infusion. This was manageable in most cases by rotation of the infusion site. However, two patients had a more severe reaction. Patient no. 4 had grade 2 local inflammation that was intolerably painful despite a reduction in drug concentration; the infusion was discontinued after 4 days. Patient no. 13 developed a grade 3 local reaction with inflammation and ulceration at one infusion site. This site was slightly edematous before commencement of the infusion as a result of lymphomatous involvement of the draining lymph nodes. Other infusion sites were less severely affected, and the complete course of treatment was delivered. Skin biopsy samples from affected sites in these two patients showed a mild perivascular lymphocytic infiltrate in the superficial dermis. Features were nonspecific and there was no evidence of vasculitis. Five patients (patients no. 11, 13, 16, 17, and 19) developed tender enlarged lymph nodes during the G3139 infusion in lymph node regions draining the skin at the infusion site. The skin and lymph node changes resolved after completion of the infusion.

In a number of patients, nonhematologic abnormalities occurred that were considered unlikely to be related to G3139 treatment. Four patients developed chest infections while on treatment (patients no. 6, 8, 11, and 20). All of these patients had advanced NHL with impaired bone marrow function. Three cases were confirmed microbiologically to be *H influenzae* (patient no. 8) or *Haemophilus parainfluenzae* (patients no. 6 and 20) infections and responded to appropriate antibiotic therapy. A microbiologic diagnosis was not possible in patient no. 11, but the symptoms resolved with empirical oral antibiotic treatment. Three patients developed circulatory abnormalities secondary to progressive lymphoma during the course of treatment. Patient no. 3 had swelling of the arms, neck, and scrotum and developed enlarging right and left pleural effusions. Patient no. 7 had superior vena cava obstruction with episodes of transient syncope. Patient no. 15 had peripheral edema and ascites associated with hypercalcemia and acute renal failure.

DLTs were observed in patients treated at doses of 147.2 mg/m^2/d and above. Of the five patients treated at a dose of 147.2 mg/m^2/d, two experienced adverse events considered to be DLTs (Table 2). These comprised grade 3 thrombocytopenia (patient no. 12) and grade 3 fever associated with grade 3 hypotension (patient no. 17). The latter event occurred within a few hours of the patient's starting the G3139 infusion and resolved rapidly once the infusion was discontinued, 48 hours after commencement. It is of note that the plasma concentration of G3139 in this patient reached an unusually high level (Table 3). He subsequently completed a second course of treatment at a reduced dose of 36.8 mg/m^2/d, without systemic toxicity. None of the three patients treated at a dose of 195.8 mg/m^2/d were able to complete the scheduled 14-day infusion. Patient no. 14 had treatment discontinued on day 8 because of a combination of fatigue, fever, and grade 4 thrombocytopenia. Patient no. 15 developed rapid disease progression associated with hypercalcemia and acute renal failure on the first day of treatment; treatment was therefore discontinued immediately. Patient no. 16 continued treatment until day 12, when the combination of grade 3 thrombocytopenia, fever, fatigue, and a generalized maculopapular rash made further treatment intolerable. The maximum-tolerated dose was therefore considered to be 147.2 mg/m^2/d (approximately 4.1 mg/kg/d).

Pharmacokinetics

Detectable levels of G3139 were observed in plasma at doses of 36.8 mg/m^2/d and above. Steady-state plasma levels were observed in the majority of patients 48 hours after the beginning of the infusion, although minor fluctu-

Table 3. Pharmacokinetic Parameters

Patient No.	Dose (mg/m² × days)	AUC (μg · mL⁻¹ · h)	C_{ss} (μg · mL⁻¹)	$t_{1/2}$ (h)	Cl [L · m⁻² · h]
6	36.8 × 14	180	0.84	7.3	8.61
7	73.6 × 14	107	0.47	17	9.62
8	73.6 × 14	184	1.03	12.5	5.60
9	73.6 × 14	343	1.59	6.2	3.00
10	147.2 × 14	454	1.43	NA	4.50
11	147.2 × 14	274	0.96	6.8	7.52
12	147.2 × 14	1,200	4.29	4.5	1.71
13	147.2 × 14	520	2.45	6.8	3.96
14	195.8 × 9	765	4.17	7.6	1.73
15	195.8 × 3	NA	7.37	NA	NA
16	195.8 × 12	789	5.36	NA	2.97
17	147.2 × 2	520	6.67	12.8	5.27
17 B	36.8 × 14	NA	0.96	NA	NA
18	73.6 × 14	261	1.02	4.55	3.94
19	110.4 × 14	657	2.23	10.2	2.37
20	110.4 × 14	627	2.11	1.33	2.47
21	110.4 × 14	616	2.60	3.38	2.50

NOTE. Parameters were evaluated with WinNonlin Pro model 202.
Abbreviations: NA, not assessable; C_{ss}, plasma steady-state concentration; $t_{1/2}$, plasma half-life; Cl, plasma clearance.

ations in the steady-state level were observed, corresponding to changes in the infusion site. Table 3 shows the pharmacokinetic parameters derived from noncompartmental analysis. The mean plasma steady-state concentration (C_{ss}) was 0.45 μg/mL (range, undetectable to 0.96 μg/mL) for the 36.8-mg/m² group, 1.03 μg/mL (range, 0.47 to 1.59 μg/mL) for the 73.6-mg/m² group, 2.43 μg/mL (range, 2.11 to 2.78 μg/mL) for the 110.4-mg/m² group, 3.16 μg/mL (range, 0.96 to 6.67 μg/mL) for the 147.2-mg/m² group, and 5.63 μg/mL (range, 4.17 to 7.37 μg/mL) for the 195.8-mg/m² group. There was a linear correlation between C_{ss} and dose ($P = .002$) (Fig 2). Apart from the dose delivered, the only factor identified in multivariate analysis to significantly affect C_{ss} was pretreatment renal function ($P = .044$, general linear model). Representative concentration-versus-time curves are shown in Fig 3 (with patients treated at the 110.4-mg/m²/d dose level as an example). The mean plasma half-life for elimination ($t_{1/2}$) was 7.46 hours (SD, ± 4.32 hours; SE, ± 1.15 hours). There was no difference in $t_{1/2}$ between dose levels (*t* test for unpaired samples), nor was there any correlation between renal function and $t_{1/2}$.

Response

All 21 patients were assessable for response on an intention-to-treat basis (Table 4). Patient no. 8 had a complete response to treatment, with resolution of all lymph node masses to less than 1 cm in diameter, and clearance of bone marrow involvement. This was accompanied by resolution of lymphoma-related alcohol intolerance, which allowed him to drink alcohol for the first time in 2 years. His remission has been maintained without further antilymphoma therapy and confirmed on repeat CT scans up to 3 years after he completed treatment (Fig 4). Two patients had a minor response to treatment. Patient no. 6 had a near partial response in her right axillary and mediastinal nodes but stable disease in her more bulky lymphadenopathy below the diaphragm. Patient no. 21 had a good partial

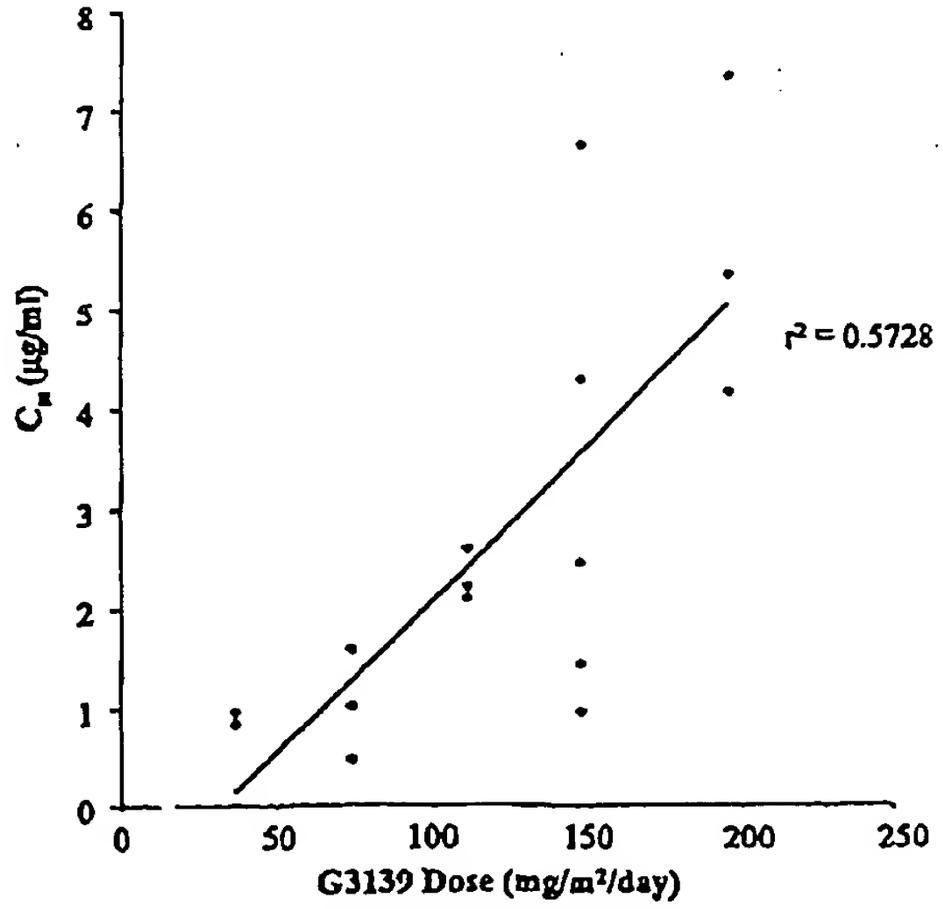

Fig 2. Administered dose of G3139 plotted against plasma C_{ss}.

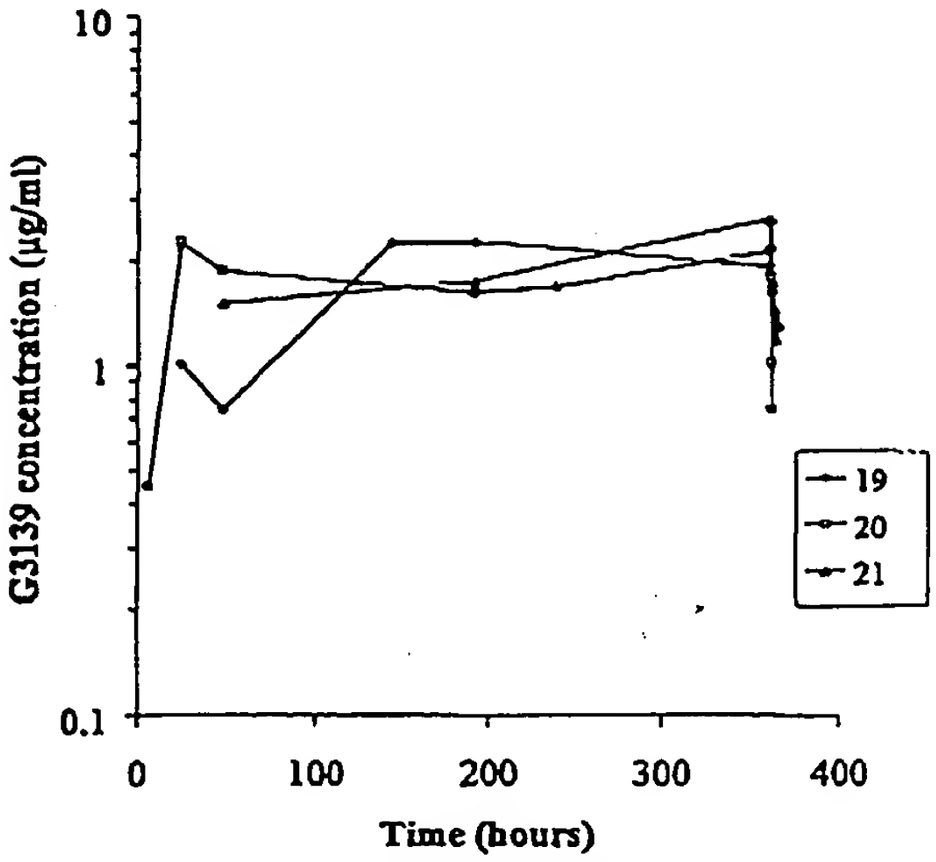

Fig 3. Plasma concentration-time curves of G3139 for patients treated at the 110-mg/m²/d dose level by continuous subcutaneous infusion.

response at nodal sites in the retrocrural area and the celiac axis and resolution of a pulmonary lymphoma deposit. However, other disease sites showed slight enlargement over the course of treatment. Eight patients had stable disease on CT scan over the 6-week assessment period (patients no. 1, 2, 11, 13, 16, 18, 19, and 20), and one patient was lost to follow-up after 3 weeks (patient no. 10), at which point he had stable disease. The remaining nine patients showed disease progression. In several cases, CT scans at the end of the 2-week G3139 infusion demonstrated enlargement of lymph nodes that subsequently returned to baseline by the 6-week assessment CT scan. In view of the observation of painful lymphadenopathy in some patients during the G3139 infusion, it is more likely that this transient nodal enlargement resulted from an inflammatory process rather than disease progression.

In addition to objective measurement of tumor response by CT scan, additional supplementary markers of response were also assessed (Table 4). Lymphoma-related symptoms were present in 10 patients before the initiation of treatment

Table 4. Clinical Response

Patient No.	Dose (mg/m²/d)	CT Response	Circulating Lymphoma Cells (× 10⁹/L) Pre	Post	% Change	B Symptoms
1	4.6	SD	NA	NA	NA	↓
2	9.2	SD	4.4	2.4	−29	NA
3	18.4	PD	0	0	NA	NA
4	36.8	PD	0.5	7.7	+1,430	NA
5	36.8	PD	0.5	1.0	+111	→
6	36.8	MR	2.2	1.1	−47	NA
7	73.6	PD	0.6	0.2	−67	→
8	73.6	CR	0	0	NA	↓
9	73.6	PD	0	0	NA	→
10	147.2	SD	14	4.5	−68	NA
11	147.2	SD	9.3	1.0	−89	↓
12	147.2	PD	0.6	0.06	−90	NA
13	147.2	SD	0	0	NA	NA
14	195.8	PD	73	65	−11	NA
15	195.8	PD	0	0	NA	NA
16	195.8	SD	80	176	+120	NA
17 B	36.8	PD	2.0	4.2	+112	NA
18	73.6	SD	0.2	0.05	−78	↓
19	110.4	SD	0.2	0.07	−66	↓
20	110.4	SD	0.3	0.1	−53	↓
21	110.4	MR	0	0	NA	NA

NOTE. Circulating lymphoma cells were assessed morphologically and quantified by counting cells on a blood film by an experienced hematologist (patients no. 2, 6, and 7) or by FACS analysis for CD19 expression (patients no. 4, 5, 10, 11, 12, 14, 16, 17, 18, 19, and 20). No circulating lymphoma cells were present in the blood of patients no. 3, 8, 9, 13, 15, and 21. B symptoms were considered reduced (↓) if the patient reported resolution or dramatic reduction in frequency of fevers or night sweats. A right arrow (→) indicates persisting B symptoms. Patients without B symptoms at any stage of participation in the trial were considered not assessable for this response parameter. Treatment-related fevers were not considered to represent B symptoms.

Abbreviations: CR, complete response; MR, minor response; SD, stable disease; PD, progressive disease; pre, before treatment; post, after treatment completion.

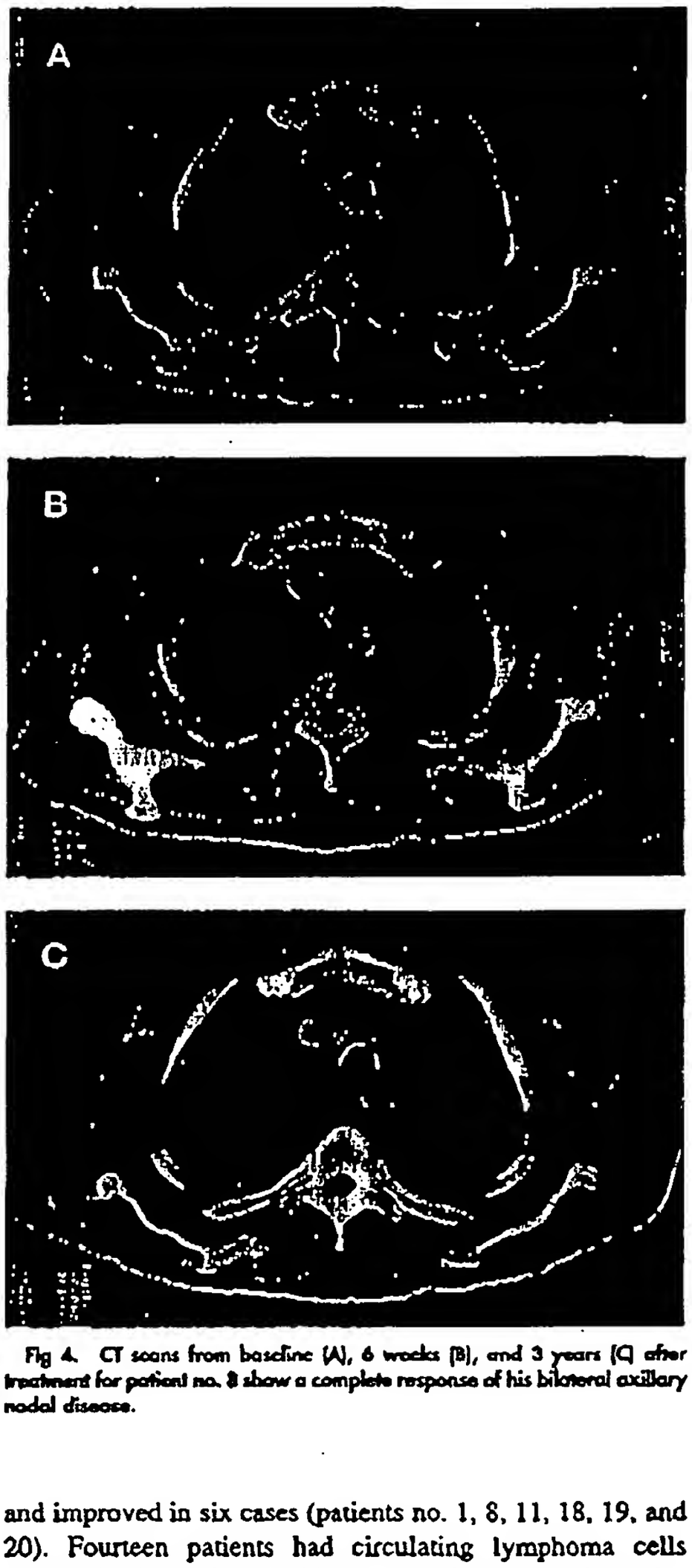

Fig 4. CT scans from baseline (A), 6 weeks (B), and 3 years (C) after treatment for patient no. 8 show a complete response of his bilateral axillary nodal disease.

and improved in six cases (patients no. 1, 8, 11, 18, 19, and 20). Fourteen patients had circulating lymphoma cells present in the peripheral blood, and 10 of them showed a reduction after treatment (patients no. 2, 6, 7, 10, 11, 12, 14, 18, 19, and 20). Two patients had normalization of an elevated serum lactate dehydrogenase level (patients no. 6 and 8). Three patients received a second course of G3139. Patient no. 2 had a further symptomatic response with the second course after the recurrence of his pruritus and night sweats between treatments. Patient no. 17 was re-treated at a reduced dose after the occurrence of DLT after 48 hours of his initial course. This was well tolerated with no systemic toxicity, but his disease progressed. Patient no. 21 received the second course by intravenous infusion because of poor tolerance of the local skin inflammation at the subcutaneous infusion sites. This was well tolerated without systemic toxicity, but again disease progressed.

Survival

Seven patients are known to have died, and one has been lost to follow-up. With a median follow-up of 23.3 months, the median overall survival for all patients was 13.4 months. In responding patients or those with stable disease, the median progression-free survival was 3.6 months. Patient no. 8 remained in continuous complete remission at 36 months after treatment, as confirmed by repeat CT scan and bone marrow examination. Patient no. 20 had no evidence of progressive disease after 7.3 months without further treatment.

Molecular Response

Bcl-2 protein levels were measured by FACS in peripheral-blood mononuclear cells (PBMCs) from patients no. 2, 6, 11, 13, 14, 16, 17, 19, and 20, in bone marrow aspirates from patients no. 2, 5, 6, 7, 14, 17, 19, and 20, and in lymph node fine-needle aspirates from patients no. 2, 4, 6, 7, 10, 11, 13, 17, 18, 19, and 21 (Table 5). It was not possible to analyze peripheral-blood or bone marrow samples from patients with no infiltration or a very low level of infiltration of these tissues by lymphoma (a minimum number of fluorescence events in the FACS analysis was required to reliably quantify Bcl-2 protein expression). A number of patients had no palpable peripheral lymph nodes from which fine-needle aspirates could be obtained. No significant changes were observed in the levels of the control HLA proteins in any samples. Bcl-2 protein was meaningfully reduced (> 15%) after treatment in samples from at least one tissue source in patients no. 2, 6, 11, 12, 13, 19, and 20. Patients no. 6, 11, and 12 had samples taken at an additional time point, after 7 days of treatment. The observed reduction in Bcl-2 protein expression was apparent at this point and was sustained until day 14. There was no reduction in Bcl-2 protein in samples from patients no. 4, 5, 7, 10, 14, 16, 18, and 21.

DISCUSSION

This study has demonstrated the feasibility of *bcl-2* antisense oligonucleotide therapy by 14-day subcutaneous

Table 5. Molecular Response: Effect of Treatment on Bcl-2 Protein Expression in Available Tumor Samples

Patient No.	PBMCs: Proportion of Lymphoma Cells (%) Pre	PBMCs: Proportion of Lymphoma Cells (%) Post	Bcl-2 Evaluation	Bone Marrow Aspirate	Lymph Node Fine-Needle Aspirate
2	56	48	↓ (24%)	↓ (29%)	→
4	22	84	NA	NA	→
5	24	39	NA	→	NA
6	33	26	→	→	↓ (47%)
7	47	19	NA	→	→
10	83	78	→	NA	→
11	48	18	↓ (24%)	NA	→
12	25	5	↓ (32%)	NA	NA
13	0	0	NA	NA	↓ (18%)
14	96	95	→	→	NA
16	89	93	→	NA	NA
17	38	79	→	→	→
18	17	6	NA	NA	→
19	20	11	↓ (36%)	↓ (12%)	→
20	15	14	↓ (15%)	→	NA
21	0	0	NA	NA	→

NOTE. A down arrow (↓) indicates a reduction in Bcl-2 protein expression from baseline to after treatment completion, with percentage change, adjusted for background fluorescence, indicated in parentheses. Changes of less than 10% were considered insignificant. A right arrow (→) indicates no reduction in Bcl-2 expression after treatment. The proportion of lymphoma cells in the peripheral blood is expressed as a percentage of total PBMCs and was assessed as described in the footnote for Table 4.

infusion in patients with advanced NHL. Treatment was universally well tolerated at doses up to 110.4 mg/m^2/d in this population. Other phase I/II studies are ongoing with G3139 administered by intravenous infusion to patients with breast, prostate, or renal cell cancers, melanoma, or other epithelial malignancies. Initial reports from these trials indicate that G3139 is tolerated with doses exceeding the maximum-tolerated dose from our study, even when combined with cytotoxic chemotherapy.[13,14] The generally advanced stage of disease, multiple prior courses of chemotherapy, and compromised bone marrow function in the patients enrolled may account for the greater degree of toxicity observed in our study. On the basis of our results, we would recommend a dose of 110.4 mg/m^2/d for further evaluation in phase II studies in patients with advanced NHL.

Although this was primarily a safety study, there was evidence for clinical antitumor activity and for specific downregulation of Bcl-2 protein in target tissues. Three patients with low-grade lymphoma had an objective reduction in overall tumor bulk after treatment, and one of them had a complete remission that has been sustained for 36 months. Although spontaneous remissions are known to occur in low-grade lymphoma, it is extremely unlikely that this would happen by chance within a few weeks of antisense therapy, particularly in heavily pretreated patients. The clinical relevance of disease stabilization over a 6-week period in patients with low-grade lymphoma is less clear. This is an indolent disease with a low proliferation rate. However, all patients had disease progression before study entry, and the median progression-free survival of 3.6 months associated with a single 2-week course of treatment is encouraging. Other trials of antisense oligonucleotides in malignant diseases have investigated repeated courses of therapy over protracted periods. In these studies, tumor responses were often not observed until several months after the initiation of treatment.[15] In the three patients with diffuse large B-cell lymphoma included in this study, the disease progressed rapidly during treatment. Although this is a small sample on which to base any firm conclusions, it is possible that the high proliferation rate of the malignant cells in this disease renders apoptosis (and therefore Bcl-2 expression) a less important factor in determining the rate of tumor growth.

One aim of this study was to demonstrate a specific antisense oligonucleotide effect on the molecular target. Preclinical studies both in vitro and in vivo have established that *bcl-2* mRNA and protein are specifically downregulated by G3139.[8,10,16] The more limited availability of tumor cells from clinical material restricted the scope of the laboratory investigations we were able to perform. FACS was selected to evaluate Bcl-2 protein levels because of its ability to provide data on individual cells. We were therefore able to restrict the analysis to Bcl-2–expressing cells; thus, we predominantly examined tumor cells. Normal mature memory B lymphocytes and T lymphocytes (CD4 and CD8) also

express Bcl-2 protein. These cells may, therefore, also have been included in the analysis in some cases, particularly in the PBMC samples. This raises the question of whether observed reductions in Bcl-2 protein resulted from a change in the proportion of tumor cells before and after treatment rather than from reduced Bcl-2 expression by tumor cells. However, one patient with significant tumor infiltration of the blood had a reduction in Bcl-2 protein in the PBMC samples (patient no. 2). Morphologic criteria and immunocytochemistry showed that the proportion of lymphoma cells in the samples analyzed did not change between time points, and these were the predominant species of cell present. Two patients had a reduction in Bcl-2 protein levels in lymph node fine-needle aspirate samples. Both patients had a diagnosis of follicular lymphoma, in which the vast majority of Bcl-2–positive cells in the involved lymph nodes are lymphoma cells. It is likely, therefore, that these observations represent a true antisense-mediated reduction in Bcl-2 protein expression. Although numbers are small, it is of interest that of the seven patients in whom a reduction in Bcl-2 expression was observed, one patient had a minor response and five had stable disease, whereas only one had progressive disease. In contrast, four of the nine patients in whom we observed no reduction in Bcl-2 expression had progressive disease by CT criteria. A further patient with unchanged Bcl-2 expression had a dramatic increase in numbers of circulating lymphoma cells, although his nodal disease remained stable. Using a dose range and schedule similar to those used in this study, another phase I/II study of G3139 by intravenous infusion, combined with dacarbazine, has demonstrated decreased Bcl-2 expression by Western blots from serial biopsies of melanoma lesions, corresponding with durable antitumor activity in some patients with drug-resistant disease.[13]

A reduction in *c-raf* mRNA levels in PBMC samples, determined by reverse transcriptase-polymerase chain reaction, was reported in patients with a variety of solid tissue malignancies treated with a *c-raf* antisense oligonucleotide.[17] Intestinal expression of intercellular adhesion molecule-1 (ICAM-1), assessed qualitatively by immunohistochemistry, was reported to be reduced in patients with Crohn's disease treated with an antisense oligonucleotide targeting ICAM-1 mRNA.[18] Patients treated with a placebo in this study showed no reduction in ICAM-1 expression. Thus, there are now four clinical trials supporting an antisense mechanism of action of phosphorothioate oligonucleotides.

Phosphorothioate oligonucleotides may also have immunologic effects,[19] and it could be speculated that the G3139 antitumor activity was related to this property rather than reduction of target protein expression. Localized activation of natural killer (NK) cell lytic activity in the draining lymph nodes was reported in mice injected subcutaneously with oligonucleotides containing the CpG dinucleotide sequence motif.[20] Activation of NK cells was dependent on the production of interleukin-12, interferon-alfa/beta, and tumor necrosis factor alpha by accessory cells. CpG-containing oligonucleotides have been shown to activate dendritic cells in the skin of BALB/c mice after local injection, which leads to a local TH1 response.[21] Our observation of skin inflammation at the subcutaneous injection site, together with painful enlargement of local lymph nodes, may represent a similar phenomenon because G3139 contains two CpG motifs. We aimed to address this point. G3139 was administered to a human lymphoma xenograft in nonobese, diabetic, severe combined immune deficient (NOD-SCID) mice that lack B or T lymphocytes or NK cells, and showed eradication of disease (F. Cotter, personal communication, June 1998). In addition, we examined intracellular expression of interleukin-2, interferon-gamma, interleukin-4, and perforin in PBMCs and serum levels of immunoglobulin E in four patients before and at the end of the G3139 infusion. There was no evidence for a systemic TH1 response or activation of NK cells as a result of treatment in these patients (unpublished data). These observations do not support an immunologic mechanism of action for G3139.

The toxicity of G3139 observed in this study seemed to be related primarily to the phosphorothioate backbone chemistry and not to a *bcl-2* sequence-specific effect. Clinical trials investigating phosphorothioate antisense oligonucleotides targeting a number of different genes have reported a spectrum of toxicity similar to that observed in this study. DLTs have included fatigue,[15,17,22,23] fever,[17] fever and hypotension,[23] and thrombocytopenia.[15,22,23] Other reported treatment-related toxicities include clotting abnormalities,[15,17,18,24] elevation of complement components,[15,17,24] elevation of hepatic enzymes,[25] and hyperglycemia.[23] It is likely, therefore, that these abnormalities are non–sequence-specific effects of the phosphorothioate oligonucleotide molecule. The mechanism underlying this toxicity is not completely understood but seems to result from the polyanionic structure of these molecules, being inhibited in vitro by the polycationic drug protamine sulfate.[26]

The potential for sequence-specific toxicity as a result of downregulation of Bcl-2 expression in normal tissues is perhaps of greater concern. Combination of *bcl-2* antisense oligonucleotides with chemotherapeutic agents may potentiate this effect, as the increased induction of apoptosis could produce significant toxicity. Bcl-2 is normally expressed in adults in a relatively restricted distribution.[27]

Transgenic mice with deletion of both copies of *bcl-2* are viable but display a variety of abnormalities, including polycystic kidneys, growth retardation, hair hypopigmentation in the second hair follicle cycle, and impaired intestinal epithelial turnover.[28,29] Hematopoiesis is normal in Bcl-2–deficient mice, and although Bcl-2 is normally expressed in megakaryocytes, both megakaryocyte number and platelet count were normal in these animals. Fundamentally, the *bcl-2* gene is not essential for survival of the pluripotent stem cell, and as such, Bcl-2 downregulation will not lead to a loss of renewal potential for the normal cell. *Bcl-x*, a homolog of *bcl-2*, seems more important in the regulation of apoptosis in hematopoietic precursors,[30] and indeed Bcl-x–deficient mice undergo massive cell death of immature hematopoietic cells and neurons and die around 13 days of gestation.[31] Early lymphoid development was also normal in *bcl-2* knockout mice, but the animals subsequently developed lymphopenia and displayed fulminant apoptosis of lymphocytes in the thymus and spleen, suggesting a dependence of mature B and T lymphocytes on Bcl-2 for survival.[28] The only clinical or laboratory abnormality observed in our study likely to have arisen from downregulation of Bcl-2 was lymphopenia. There have been no other reports of lymphopenia in clinical trials of phosphorothioate oligonucleotides, but nine patients had a transient reduction in lymphocyte count during the 2-week period of treatment with G3139. Of these, Bcl-2 expression was assessable in five patients' PBMCs and reduced in three. An alternative explanation for the occurrence of lymphopenia in these patients with advanced NHL is progressive bone marrow involvement by disease. However, this would not explain the temporal relationship to treatment we observed, and lymphopenia could therefore be a reversible sequence-specific effect of G3139.

The C_{ss} level of G3139 observed in patient no. 8, who showed a complete response, was consistent with that measured in the mouse after continuous infusion at therapeutic doses.[12] Indeed, the pharmacologically active plasma level, associated with a reduction in target protein expression, seems to be consistent between mice and humans at approximately 1 μg/mL (Fig 5).[12] This suggests that the therapeutic window for this molecule is wide in comparison with conventional cytotoxic agents, as DLT was only observed when plasma levels exceeded 4 μg/mL. At a given dose level, up to 6.9-fold variations in C_{ss} were observed (eg, 0.96 for patient no. 11 and 6.67 for patient no. 17, both treated at 147.2 mg/m²/d). However, patient no. 11 required multiple changes in infusion site because of skin toxicity that could potentially have affected the C_{ss} levels. Furthermore, no patient treated at a dose of 110.4 mg/m²/d or below achieved a plasma level of G3139 in the range associated

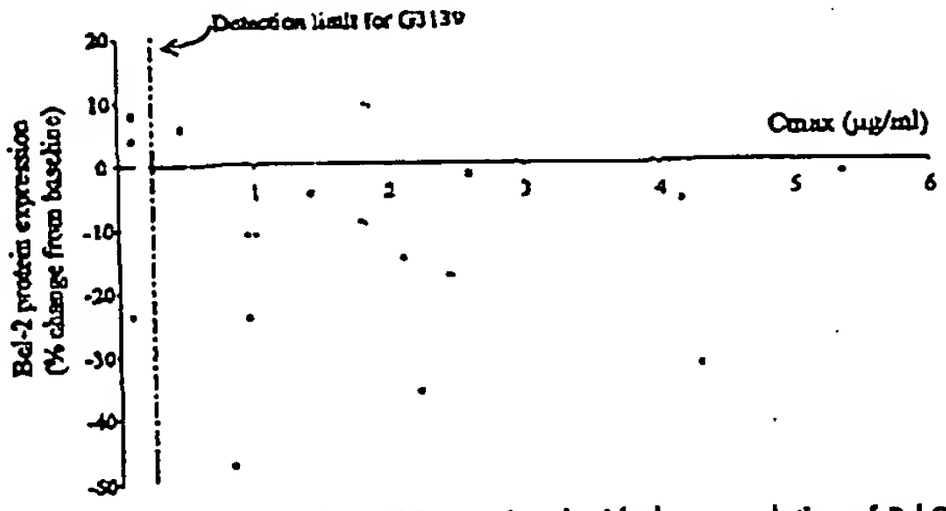

Fig 5. Plasma level of G3139 associated with downregulation of Bcl-2 protein. Changes in Bcl-2 protein in patients with plasma G3139 levels below the detection limit of our assay (0.25 μg/mL) are represented by open symbols (○: patients no. 2, 5, and 7).

with toxicity, which suggests that treatment with these doses should be consistently safe. Although it was difficult to evaluate absorption in our pharmacokinetic analysis because of the complexity of the administration and elimination, the pharmacokinetics were linear with increasing doses suggesting that the absorption was consistent whatever the dose. This is contrary to what has been described in the rat, in which the bioavailability of a 20-mer phosphorothioate after subcutaneous administration has been shown to increase with dose.[32] In a study in which G3139 was infused intravenously, C_{ss} plasma levels after intravenous administration were very similar to those observed in our study, which indicates that G3139 is well absorbed by the subcutaneous route.[14] The $t_{1/2}$ of G3139 (7.2 hours) was different from that reported for other phosphorothioate oligonucleotides in the clinical setting, where $t_{1/2}$s of 30 minutes and 26 hours have been reported.[24,33,34] However, these studies were performed using short intravenous infusions, and it is possible that both the long infusion and the subcutaneous route affected the terminal $t_{1/2}$, as was observed in the preclinical model.[32] The variation in $t_{1/2}$ observed between patients was unrelated to dose or renal function and may have reflected differences in rate of absorption from the subcutaneous compartment after the end of the infusion. The overall plasma clearance, however, correlates well with what has been reported in other studies with short intravenous infusion.[33]

There is great potential for the further development of *bcl-2* antisense oligonucleotides in the treatment of malignant disease. One of the most interesting possibilities is their use as chemosensitizing agents, both in NHL and in other malignancies characterized by Bcl-2 overexpression. This principle has been demonstrated in vitro and in vivo with several human tumor models, including lymphoma,[9,35]

breast cancer,[36] small-cell lung cancer,[37] prostate cancer,[38] and melanoma.[16] Clinical trials are now underway in several malignancies besides NHL, using G3139 alone or in combination with cytotoxic agents. Our results suggest that advanced-low grade lymphoma is amenable to *bcl-2* antisense oligonucleotide therapy. Based on the results from this phase I study, a phase II trial is now in progress at the Royal Marsden Hospital using G3139 in combination with standard cytotoxic regimens for patients with relapsed, chemotherapy-resistant NHL.

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Targeting the Expression of Anti-Apoptotic Proteins by Antisense Oligonucleotides

Nicholas Delihas*

Department of Molecular Genetics and Microbiology, School of Medicine, SUNY, Stony Brook: Stony Brook, NY 11794-5222 USA

Abstract: Antisense oligonucleotide (ASO) biotechnology has been widely used to inhibit the expression of proteins involved in human disease. ASOs are designed to bind messenger RNA transcripts via Watson-Crick base-pairing and inhibit synthesis of targeted proteins. These proteins include protein kinases, growth factors, glutamate receptors, anti-apoptotic proteins, and proteins involved in genetic disorders. Non-mRNA targets such as the RNA component of the telomerase enzyme are also being explored. Pre-clinical and clinical trials using ASO biotechnology have progressed with standard ASOs such as phosphorothioates, but newer ASO analogs are rapidly being developed with the idea of enhancing specificity and biological activity. A current major research thrust is the design and testing of antisense oligonucleotides as anti-cancer drugs. The primary focus of this review is an analysis of recent uses of ASO biotechnology to inhibit anti-apoptotic gene expression in tumor cells.

BACKGROUND

Antisense oligonucleotides (ASOs) are being developed to target specific genes for therapeutic purposes. ASOs are synthetic polymers of approximately 20 nucleotides (nt) in chain length [1]. These molecules are designed to be complementary to target RNAs. They bind via Watson-Crick base-pairing and inhibit RNA function. Most targets are messenger RNAs where gene expression is inhibited at the post-transcriptional level. This can involve interference with mRNA processing, mRNA transport, or translation of the message. Telomerase RNAs, which form templates for reverse transcription and addition of DNA sequences to ends of chromosomes, have also been used as targets [2]. ASOs function by either inducing cleavage of the target RNA by RNase H, an enzyme that cleaves the RNA of a DNA/RNA duplex, or blocking the binding of the target RNA to another molecule via steric hindrance. Additionally, RNase L, an endonuclease that cleaves single-stranded RNA, is present in higher organisms and is activated by 2',5'-oligoadenylates, termed 2-5A [3]. 2-5A moieties have been attached to antisense oligonucleotides to direct RNase L cleavage of target RNAs [4-6]. ASOs have also been engineered to bind to double- stranded DNA and form triple helices. It has been shown that formation of a triple helix will inhibit expression of a specific target gene [7].

NEW CONCEPTS IN THE DESIGN OF ANTISENSE OLIGONUCLEOTIDES

Most studies utilizing antisense biotechnology have been done with phosphorothioate (PS) oligonucleotides [8] (Fig. 1). These analogs have a sulfur substituted for an oxygen in a non-bridging oxygen atom of the phosphodiester backbone. This renders the oligonucleotide resistant to nucleases. Phosphorothioates however tend to bind non-specifically to proteins [9-11]. These non-specific interactions can complicate the mode of biological action of the PS ASO and can produce toxic effects as well.

*Address correspondence to this author at the Department of Molecular Genetics and Microbiology, School of Medicine, SUNY, Stony Brook, Stony Brook, NY 11794-5222 USA; Tel: 631 632 8779; Fax: 631 632 9797; E-mail: nicholas.delihas@sunysb.edu

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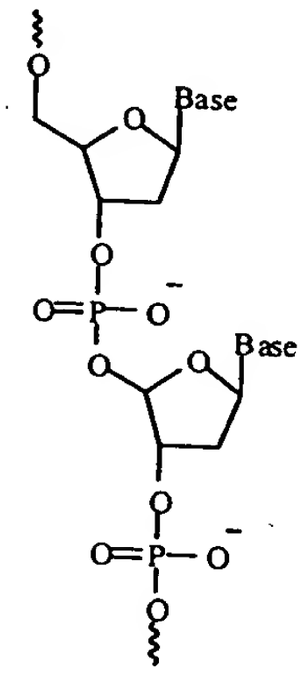

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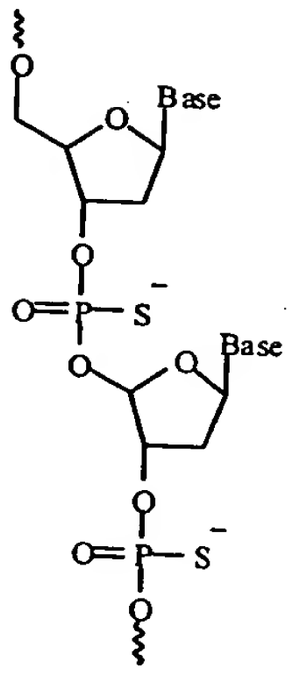

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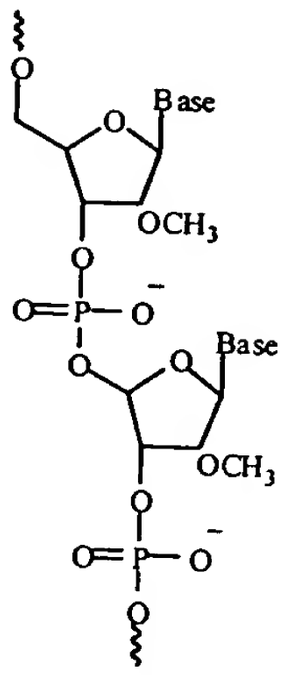

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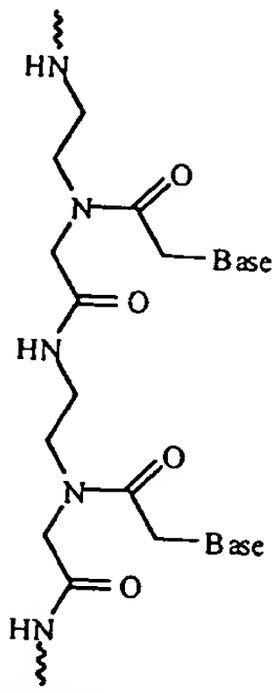

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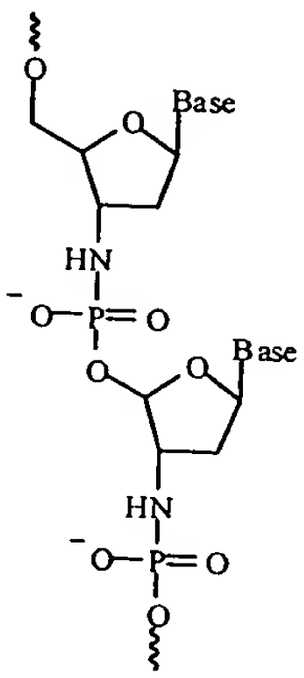
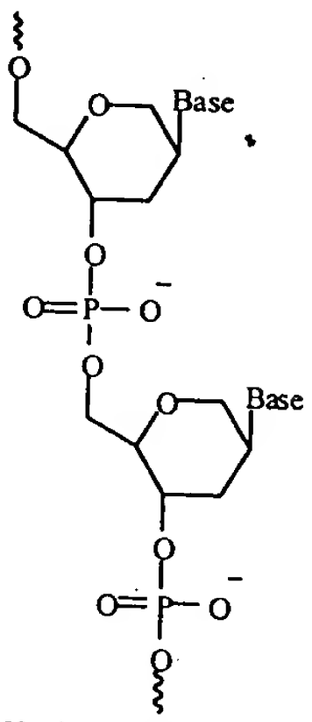
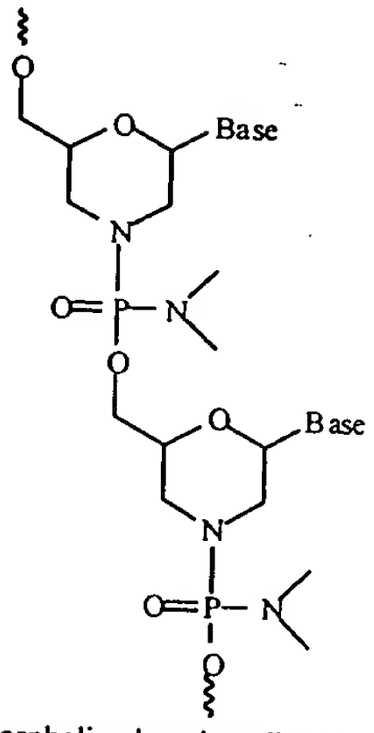


N3'-P5' phosphoramidate oligonucleotide chain (PN)



Hexitol nucleic acids oligonucleotide chain (HNA)



Morpholio phosphorodiamidate oligonucleotide chain (MF)

Fig. (1). Antisense oligonucleotide analogs currently used and /or being developed for antisense therapeutics.

New analogs are rapidly being developed in an attempt to increase stability, target selectivity, and biological activity, and to also minimize toxicity in vivo. Modifications of the 2' position of the sugar moiety and/or phosphodiester backbone substitutions have yielded antisense molecules that form highly stable duplexes with target RNAs. Mixed backbone oligonucleotides containing a mixture of phosphorothioate and 2'-*O*-methyl ribonucleoside residues have proven to have a high affinity for RNA and to display fewer side effects in vivo than PS ASOs [12]. Other newly developed analogs are N3'-P5' phosphoramidate backbone-modified oligonucleotides (Fig. 1). These bind very tightly to RNA, are nuclease resistant, and may act solely via antisense inhibition based on correlation of decreased expression of the target RNA with formation of the modified- ASO/target RNA duplex [13]. Like the phosphoramidates, hexitol oligonucleotide analogs are part of the family of backbone-modified polymers that bind tightly to RNA [14] (Fig. 1). Hexitol nucleic acids are less active but more selective than PS ASOs [14]. Morpholino oligonucleotides and peptide nucleic acids (Fig. 1), which represent additional classes of backbone-modified oligonucleotide analogs, have high sequence specificity and display a minimum of non-antisense interactions [15, 16]. Novel unmodified phosphodiester oligonucleotides consisting of "circular ribbon" covalently closed structures and containing multiple antisense sequences have a high stability in serum and are

highly active in inhibiting gene expression [17, 18]. 3'-amino-modified phosphodiester oligonucleotides have been designed to bind to double-stranded DNA instead of RNA and have been shown to specifically inhibit gene expression [7]. 2',5'-oligoadenylates have been attached to antisense oligonucleotides (2-5A ASO) to direct RNase L against the telomerase RNA. The 2-5A ASOs are highly active inhibitors of telomerase activity and may be useful in treatment of different types of cancers.[4-6]. The newly developed DNA analogs called locked nucleic acids (LNAs) form highly stable duplexes with DNA and RNA, are substrates for RNase H, appear to display no toxicity, and have high biological activity in vivo when tested in rat brain. [19]. Non-canonical base-pairs and 'imperfect" base-pairings may also be considered in the design of antisense molecules to improve the stability of an oligonucleotide/target RNA duplex [20]. These interactions play a crucial role in stabilizing intra-and inter- polynucleotide chain associations found in natural RNAs.

There is thus a rich variety of nucleic acid analogs that are being developed to improve antisense biotechnology. We awąit further studies on the efficacy of these exciting new compounds.

DISEASE TARGETS OF ANTISENSE OLIGONUCLEOTIDES

Antisense technology has been used to study gene function and investigate possible therapeutic use [21]. (Table 1) lists current examples of disease-related genes targeted by ASO in cells in culture and/or animal models. Although the works cited are not all inclusive, the list provides an idea of the variety of genes and diseases that are being targeted by ASOs. A large number of cancer-related genes are also being targeted by antisense molecules (Table 2). In most of the examples shown, ASO were designed to bind to messenger RNAs but the telomerase RNA has also been a target in different types of tumors (Table 2).

Table 1. Examples of Diseases Targeted by Antisense Oligonucleotides*

Disease or condition	Gene target	Reference
Neurodegenerative disorders	huntingtin gene	[22]
Crohn's disease and other inflammatory diseases	ICAM-1	[23, 24]
Inflammatory Disorders	interleukin-1 receptor- associated kinase	[25]
Epilepsy	glutamate receptors	[26]
Hypertension	angiotensin type 1 receptors	[27]
Intimal hyperplasia	platelet-derived growth factor receptor-beta	[28]
Neointimal hyperplasia	proliferating nuclear antigen and cdc 2 kinase	[29]
Autologous or allogenic transplantation	VLA-4	[30]
Heart allograft survival	C-raf	[31]
Beta-thalassemia	beta-globin gene	[32, 33]
Cystic fibrosis	transmembrane conductance regulator gene	[34]
Hepatitis	fas	[35]

*In vitro and/or animal experiments

Table 2. Examples of Cancer-Related Genes Targeted by Antisense Oligonucleotides

Gene target	Cancer	Reference
ras	hepatocellular carcinoma	[36]
protein kinase A	breast	[37, 38, 39]
protein kinase A	colon	[39, 40]
protein kinase A	lung	[39]
Id-1	breast	[41]
AlphaV integrin subunit	breast	[42]
androgen receptor	prostate	[43]
testosterone-repressed prostate message-2	prostate	[44, 45]
c-myc	prostate	[46]
c-myc	small cell lung	[47]
c-myc	leukemia	[7]
c-myb	leukemia	[18]
telomease	prostate	[5]
telomerase	intracranial malignant gliomas	[4]
telomerase	ovarian	[6]
mouse double minute 2	colon	[48]
dihydrofolate reductase	leukemia	[49]
survivin	lung adenocarcinoma	[50]
intrleukin-10	tumors in general	[51]

For the most part, the studies cited in Tables 1 and 2 show dose– and time-dependent action of the ASO. Sense and/or scrambled oligonucleotide sequences have been used as controls. Methods of ASO delivery have included encapsulation with liposomes, but current efforts are focused on exploration of novel methods of delivery to increase efficiency of internalization into cells [52-55]. These include the use of vehicles that by-pass the endocytic pathway of entry into cells. In addition, new information is evolving on intracellular location of oligonucleotides such as the apparent shuttling of PS oligonucleotides from the nucleus to the cytoplasm and back [56]. Microarray gene expression analysis [57] to determine if changes occur in expression of genes other than the ASO- targeted gene has not been used yet, but this needs to be part of standard controls in future studies on ASO inhibition.

One antisense oligonucleotide, Formiversin, has been approved by the Food and Drug

Administration for the treatment of cytomegalovirus-induced retinitis in AIDS patients [58]. However clinical trials with other diseases involving systemic delivery of ASO have yielded mixed results with varied efficacy [8, 21, 59, 60]. Current trials are predominantly in Phase I or Phase II and have been performed with PS ASOs or mixed backbone analogs. The exploration of newer ASO analogs which have increased stability, target specificity, biological activity, and low toxicity as was mentioned above may provide the basis for expanded clinical trials.

BACKGROUND ON APOPTOSIS-RELATED GENES

Apoptosis is defined as programmed cell death where damaged cells are eliminated by the organism [61]. Apoptosis can be initiated by two pathways, 1) The caspase signaling pathway involving the TNF-related cell surface receptors [62], and 2) via mitochondrial dysfunction [63, 64]. The roles of various proteins in these apoptotic pathways are currently being ascertained.

The *bcl-2* (B-cell lymphoma/leukemia 2) gene locus was discovered in a B-cell leukemia cell line during the analysis of chromosomal translocations [65]. The *bcl-2* family consists of at least 20 related genes [66]. Some of these genes encode anti-apoptotic proteins while others encode pro-apoptotic proteins. Several Bcl-2 family proteins are part of the outer membrane of mitochondria and either form channels or regulate outer membrane pore proteins formation [64]. These pore proteins regulate cytochrome *c* release from mitochondria. Release of cytochrome *c* can cause mitochondrial dysfunction and subsequent cell death.

Bcl-2 family proteins are thought to play a major role in neoplastic development [66]. Many tumor cells express anti-apoptotic proteins which allows these cells to persist. In addition, these proteins are overexpressed in some cancers which can result in resistance to chemotherapy [67-74]. Thus anti-apoptotic Bcl-2 family proteins have been targets in numerous studies utilizing antisense biotechnology.

The remainder of this review focuses on the effects of antisense oligonucleotides on the expression of four anti-apoptotic *bcl-2* gene family members: *bcl-2*, *bcl-x_L*, *A 1* and *mcl-1*. The importance of these Bcl-2 family proteins in arresting apoptosis in different tumor cells has been investigated. These studies and have set the ground-work for testing of ASOs as anti-cancer drugs in human trials. Although numerous investigations show impressive results in tumor cell inhibition, analyses of possible changes in levels of apoptotic proteins other than the target protein have not always been performed. This is important to verify the selectivity of the ASO employed.

BCL-2 GENE FAMILY PROTEINS AS ANTISENSE TARGETS

Bcl-2

Initial experiments on the inhibition of *bcl-2* expression by ASO were by Reed et al [75]. Both unmodified and phosphorothioate oligonucleotides were used against bcl-2 in a human leukemia cell line. Levels of Bcl-2 protein and cell viability were reduced by both antisense oligonucleotides. Phosphorothioate ASO technology was later used to confirm the central role of Bcl-2 in prolonging the survival of acute myeloid leukemia (AML) cells and to associate high levels of Bcl-2 with resistance to chemotherapy [76]. In these experiments exposure to antisense phosphorothioate accentuated the killing of AML cells by chemotherapeutic agents. These and other studies help set the ground work for subsequent experiments and clinical trials using ASO to inhibit *bcl-2* in cancer cells [77]. Recent uses of ASO targeted against *bcl-2* expression continue to validate, fine tune and expand the potential for therapeutic uses of ASO in clinical trials. Several examples are provided.

Small cell lung cancer cells have a high expression of *bcl-2* [74]. Similar to other cancers, small-cell lung cancer cells can display resistance

to chemotherapeutic agents. To investigate possible inhibition by antisense oligonucleotides, thirteen ASOs that target different parts of the bcl-2 message were tested [78]. These were delivered with cationic lipids to facilitate uptake. One ASO in particular, designed to target a segment of the coding region of the bcl-2 mRNA was found to be highly effective in decreasing *bcl-2* mRNA and Bcl-2 protein levels in these cells [78]. Other ASOs that target the initiation or termination translational sites on the mRNA were also tested but were not found to be effective. The inactive ASOs may not be able to base pair with target sequences because of possible secondary/tertiary structural shielding of targeted mRNA sequences. The authors suggest that antisense oligonucleotide therapy may provide a viable alternative to treating chemoresistant small-cell lung cancers and that further study is warranted.

The development of hepatocarcinoma in rats was inhibited by using a novel combination of anti-Bcl-2 PS ASO encapsulated in liposomes and in vivo electroporation to maximize entry of ASO into target cells [79]. Also, attempts were made to maximize the specificity of the antisense PS. Nucleotides 13-27 just downstream of the translation initiation site of the *bcl-2* mRNA were targeted particularly because these sequences are not in other bcl-2 family member proteins which share homologous domains. *bcl-2* mRNA levels were shown to diminish and the apoptotic index increased with PS antisense oligonucleotide treatment, however changes Bcl-2 protein or other anti-apoptotic protein levels were not examined in this study.

Chemotherapy is largely ineffective in advanced prostate cancer. It was previously shown that Bcl-2 levels are high in human prostate cancer cells [80, 81]. Thus antisense ASOs have been used in attempts to inhibit *bcl-2* expression in prostate cancer tissues and induce apoptosis. A phosphorothioate ASO directed against the translational initiation region of the bcl-2 mRNA and a two base mismatched control were used in a study with the transplantable Shionogi tumor in mice [82]. It was demonstrated that the PS ASO will act synergistically with the chemotherapeutic agent paclitaxel in reducing *bcl*-2 mRNA and Bcl-2 protein levels as well as inhibiting tumor cell growth in vivo. Northern and western blots were used to monitor changes in *bcl-2* mRNA and Blc-2 protein levels. The authors suggest a possible initiation of clinical trials using combined antisense oligonucleotide/paclitaxel treatment in patients with advanced prostate cancer.

Inhibition of *bcl-2* expression by ASO in acute myeloid leukemia (AML) cells induces apoptosis [83]. In subsequent experiments, more dramatic results were obtain with the use of liposomes for administration and better uptake of anti- bcl-2 ASO [84]. Importantly, the quantitation of other anti-apoptotic proteins was performed in this study which is one of the first to look at possible effects on other Bcl-2 family member proteins with ASO directed against the Bcl-2 protein. The results show several interesting findings. 1) The inhibition of *bcl-2* expression in established AML cell lines results in apoptosis in the presence of over-expression of two other anti-apoptotic proteins, Bcl-X_L and Mdl-1. This underscores a possible role of Bcl-2 as the major anti-apoptotic factor in AML cells. 2) The antisense oligonucleotide acted in synergy with cytosine-arabinoside (ara-C) in cell killing, even in cells resistant to ara-C alone. 3) ASO also decrease Bcl-2 levels and induced apoptosis in primary AML cells in approximately 60% of samples from patients. However down-regulation of the *bcl-2* gene was achieved primarily in AML cells expressing low levels of Bcl-2. The authors concluded that acute myeloid leukemia patients who have a low baseline of Bcl-2 in their AML cells would be best suited for clinical trials using antisense oligonucleotides.

Bcl-2 is also predicted to play an important role in the survival and chemo-resistance of breast cancer cells. Human breast cancer cell line MCF-7 and a subline of this strain, LCC2 display very different phenotypic features [85]. MCF-7 is a prototype of early breast cancer cells that are estrogen- dependent and sensitive to chemotherapeutic agents, including tamoxifen. LCC2 is representative of later stage breast cancer cells which are tamoxifen resistant and estradiol-independent for growth and are more resistant to

induction of apoptosis by chemotherapeutic agents. In addition, Bcl-2 levels are much higher in LCC2 cells relative to MCF-7. By using one concentration of PS ASO, the growth of MCF-7 cells was found to be inhibited by 85%, whereas growth of LCC2 cells was unaffected. Assays were also performed for relative change in Bcl-2 levels in both strains after antisense oligonucleotide treatment. A large decrease in Bcl-2 levels was seen in MCF-2 cells but only a minor reduction was observed in LCC2 cells. Quantitation of Bcl-2 protein and mRNA levels/cell as a function of increasing concentrations of ASO are essential in this type of study. It may be that sufficient ASO can not be delivered to cells to reduce Bcl-2 when Bcl-2 levels are above a certain threshold value. Relevant to the conclusions drawn by the authors, it may be that very high levels of Bcl-2 account for the resistance of the LCC2 strain to chemotherapeutic agents.

In a related study with breast cancer cell lines, phosphorothioate ASO G 3139 (Genta, Inc), an oligonucleotide that is currently used in various pre-clinical and clinical trials, was found to inhibit *bcl-2* expression in both MCF-7 cells and in strain MDA435/LCC6 [86]. The latter strain is estrogen receptor negative. Successful reduction of *bcl-2* expression to approximately 80% was obtained in both cell lines and this reduction was associated with about an 80% decrease in viable cells compared to untreated samples. On the other hand, no synergy was found between the ASO and cytotoxic chemo-agents. The authors conclude that other factors play a role in cytotoxic-induced apoptosis. Importantly, the expression of other *bcl-2* family genes were also analyzed in this study. In MCF7 the ASO designed to inhibit *bcl-2* mRNA did not change levels of Mcl-1, Bax, or Bak but did decrease Bad and Bclx. In MDA435/LCC6 Bad was diminished but Mcl-1 and other Bcl-2 family proteins were unchanged. Thus G3139 does not alter the expression of the related anti-apoptotic protein Mcl-1. However it does affect the expression of other *bcl-2* family genes.

The Epstein-Barr Virus (EBV) is associated with post-transplant lymphoproliferative disorders, Burkitt's lymphoma, and other malignancies. Cells from these disorders as well as EBV EB - immortalized lymphoblastoid B cells (LCL) synthesize Bcl-2 and other anti-apoptotic proteins due to the expression of the viral-specific protein LMP-1 [87, 88]. To determine if Bcl-2 can be reduced in LCL cells, PS ASO G3139 (Genta, Inc.), which targets the first six codon of the human bcl-2 mRNA sequence, was delivered via liposomes to these cells [89]. Western blots were use to assay for changes in Bcl-2 protein. The ASO decreased *bcl-2* expression, inhibited cell proliferation, and induced apoptosis. This work and a subsequent study on antisense inhibition of LMP-1 expression in LCL cells [90] have set the ground work to explore a possible therapeutic value of ASO in EBV-associated cancers. A related study using an EBV-specific LMP-1 gene expressed in EBV-negative lymphoma cells showed that *bcl-2* can be induced in this system by the LMP-1 protein [91]. Interestingly, increases in Bcl-2 protein occur before an increase is seen in *bcl-2* mRNA thus indicating that there are translational controls of *bcl-2* mRNA expression in these cells.

Bcl-2 Clinical Trials

Positive results with *bcl-2* suppression in animal models have led to clinical trials. These were initiated with 9 patients with non-Hodgkin lymphoma using Bcl-2 as a target and PS ASO G3139 for antisense therapy [77]. The pharmacokinetics of ASO G3139 were previously analyzed in mice [92]. In the clinical trials, samples from approximately 40% of patients showed reduced Bcl-2 levels and clinical improvement in two patients who showed a shrinkage in tumor size with ASO therapy [77]. A follow-up clinical trial using 21 patients enabled a further evaluation of the safety and pharmacokinetics of G3139 [93]. No signs of systemic toxicity were found. Clinical results were mixed, however. One patient showed complete remission up to approximately 3 years after treatment, eight patients displayed stable disease with a marked decrease in circulating lymphoma cells, and nine patients had progressive diseases after treatment. Evidence for antisense inhibition

of *bcl-2* expression in tumor cells and the partial anti-tumor activity of ASO G3139 in this cohort of patients suggests that further clinical studies with G3139 and non-Hodgkin lymphoma are warranted.

The same PS ASO, G3139 was used in a different clinical trial with 14 patients having malignant melanoma [60]. Antisense oligonucleotide was administered in combination with the chemotherapeutic agent dacarbazine. One purpose of the study was to evaluated toxicity and side-effects. No non-hematological side effects were found but the treatment resulted a small amount of hematological abnormalities. Positive therapeutic effects were found. Bcl-2 protein levels were reduced in 10 out of 14 patients after ASO treatment. This demonstrates that the ASO was effective in limiting *bcl-2* expression in melanoma cells of most patients. A concomitant increase in apoptosis in melanoma cells was also found. The clinical findings showed that 6 patients showed a positive anti-tumor response, two were partial responses, and three displayed a minor response. The average survival of all patients in the study was greater than 12 months whereas the average for a similar cohort of patients is 4-5 months. Phase III clinical trial on patients with malignant melanoma using a combination of G3139 ASO and dacarbazine are planned.

Bcl-X_L

bcl-x_L is also an anti-apoptotic gene. Recent studies have addressed the feasibility of using ASOs to inhibit *bcl-x_L* expression and induce cytotoxic effects and apoptosis in cancer cells.

Overexpression of *bcl-x_L* in pancreatic cancers correlates with shorter patient survival. High Bcl-X_L levels are predicted to contribute to the viability of pancreatic cancer cells in patients [69]. Support for a role of Bcl-X_L as an important anti-apoptotic factor in pancreatic cancer cells was obtained using ASO regulation [94]. By using antisense oligonucleotides to inhibit *bcl-x_L* mRNA expression in pancreatic carcinoma cell lines, Bcl-X_L was found to protects these cells from apoptosis that is mediated by the TNF-related signal transduction pathway, i.e., the receptor-mediated death pathway [94].

In primary adenocarcinoma of the colon, *bcl-xL*, as opposed to *bcl-2*, is overexpressed [95]. Standard chemotherapy treatment of colon cancers is with 5-fluorouracil, however late stage metastatic tumors respond poorly to the drug. High level of Bcl-X_L are believed to be associated with resistance to 5-fluorouracil. A phosphorothioate ASO was designed to inhibit *bcl-x_L* expression in a colon cancer cell line [95]. This PS ASO, which targets the start codon of the *bcl-x_L* mRNA, was selected empirically from a set of ASO that target coding and non-coding regions. Bcl-x_L protein levels were significantly reduced by the ASO in a dose-dependent fashion. Assays for proteins Bcl-2 and Bax showed that these proteins were not affected by antisense treatment. Apoptosis was increased 30-fold with ASO treatment in these cells. Significantly, ASO exposure also increased the sensitivity of the colon cancer cell line to 5-fluorouracil. The data warrant further experiments on colon cancer cell treatment with a combination of this ASO and 5- fluorouracil.

bcl-x_L is overexpressed in lung adenocarcinoma [96] and both *bcl-2* and *bcl-x_L* are overexpressed in small- cell lung cancer cells [74, 96]. Both types of lung cancer cells were used to determine if inhibition of *bcl-x_L* will induce apoptosis [97]. A 2'-methoxy-ethyl ASO was designed to target specific sequences in the coding region of the *bcl-x_L* mRNA which are not present in the pro-apoptotic *bcl-x_S* mRNA. The results show that ASO treatment induced apoptosis in lung-adenocarcinoma cells but not in small-cell lung cancer cells even though Bcl-x_L was inhibited in both cell lines, i.e., 90% inhibition in adenocarcinoma cells and 70% in small-cell lung cancer cells. The possible resistance of small-cell lung cancer cells to apoptosis was attribute to the high levels of Bcl-2 in these cells. In previous work an ASO targeted *bcl-2* mRNA was found to be effective in decreasing Bcl-2 levels against small-cell lung cancer cells [78].

A logical follow up to these experiments is described by Zangemeister-Wittke et al [98].

ASOs, termed bispecific, were designed to target unique sequences present in both Bcl-2 and bcl-xL but not in other bcl-2 family member genes including the pro-apoptotic *bcl-x$_S$*. By Western blots, one of the bispecific antisense oligonucleotides was found to be particularly active in decreasing both targeted genes: *bcl-2* by about 22% and *bcl-x$_L$* by about 18% of untreated controls. In addition, both small-cell lung cancer cells and non-small cell lung cancer cells were found to exhibit substantial cytotoxic effects and undergo induced apoptosis. The authors suggest that bispecific oligonucleotides be considered for clinical trials in lung cancer therapy.

In a similar vein, both *bcl-2* and *bcl-x$_L$* were inhibited in an additive fashion by the use a combination of two antisense oligonucleotides targeted against each gene in an androgen-dependent mouse Shionogi tumor model [99]. A significant increase in apoptosis was obtained with the simultaneous administration of the two ASOs as opposed to use of these antisense molecules separately. Thus dual antisense targets appear to extend the potential of antisense-induced apoptosis in tumor cells.

The overexpression of *bcl-x$_L$* in primary untreated breast carcinomas has been reported [71]. In breast cancer cell line MCF7, both anti-apoptotic proteins Bcl-x$_L$ and Bcl-2 are overexpressed. To determine effects of Bcl-X$_L$ reduction on apoptosis in these breast carcinoma cells, a 2'-0-methoxy-ethoxy oligonucleotide was used to target the *bcl-x$_L$* mRNA at positions downstream and distal to the initiation start site [100]. The targeted sequences were chosen because they are not present in the pro-apoptotic *bcl-x$_S$* mRNA. Results showed that Bcl-X$_L$ protein levels decreased by approximately 75% and induction of apoptosis occurred in approximately 50% of MCF7 cells treated with the ASO. Of significance is that in spite of the overexpression of *bcl-2* in these cells, reduction of Bcl-X$_L$ by ASO can induce apoptosis. However a comparison with the effect of Bcl-2 reduction and decrease in cell growth and induced apoptosis in the same cell line (MCF7) [85, 86] indicates that more needs to be understood vis-à-vis the complex roles of these interrelated proteins in apoptosis. Zapata et al [101] showed that expression of Bcl-2 family proteins in different breast cancer cells is complex. Relative levels of both pro- and anti-apoptotic Bcl-2 family proteins need to be considered to understand the balance towards or against apoptosis, e.g., low levels of Bak, the pro-apoptotic protein are of importance to the malignant progression of breast cancers [102]. In this context it is of interest that oligonucleotides that inhibit pro-apoptotic gene expression have been successfully used to induce apoptosis in breast cancer cells.

A1 FUNCTION

The anti-apoptotic gene *A1* was discovered in hemopoietic cell lines as a gene that is transiently expressed during induction of differentiation and proliferation of these cell lines [103]. Transfection of *A1* into a myeloid precursor cell line results in the prolonging of cell survival [73]. Also, during B cell development in bone marrow, *A1* is overexpressed approximately 10-fold as these cells differentiate into long-lived mature B cells [72]. In T cells *A1* is also developmentally regulated and may play a role in cell viability [104]. A1 is found in high levels in stomach cancers [105] and is over-expressed in Epstein-Barr Virus-immortalized B cells and Burkitt's lymphoma cell lines where synthesis of *A1* mRNA levels are regulated by the EBV-specific protein LMP1 [91].

TNFα is a cytokine that participates in the inflammation process of endothelial cells. It is also a major factor in the induction of apoptosis in tumor cells. The transfection of *A1* into human microvascular endothelial cells protected these cells from apoptosis when challenged by TNFα [106]. To further elucidate a role of *A1* as a cytoprotective gene, Ackermann et al [107] used a 2'-O- Methoxyethyl chimeric oligonucleotide directed against the 3' UTR region of the *A1* mRNA to inhibit expression of A1 in endothelial cells challenged with TNFα with the rationale that TNFα induces apoptosis but that anti-apoptotic genes need to be suppressed. Reduction of *A1* mRNA was to basal levels (assays for A1 protein

expression, however, were not measured). Interestingly, inhibition of *A1* mRNA did not show susceptibility of endothelial cells to undergo apoptosis in response to TNFα. The authors conclude that A1 is not a major anti-apoptotic factor in endothelial cells challenged by TNFα. In a related study, Duriez et al [108] found that A1 can delay but not prevent apoptosis of endothelial cells over time. Thus the role of A1 in apoptosis induced by TNFα in endothelial cells is complex and A1 may play a secondary role to the mitochondria-independent caspase pathway in apoptosis [108].

MCL-1 FUNCTION

Kozopas and co-workers [109] discovered the *mcl-1* gene and showed that it has sequence similarity to the *bcl-2* gene. Mcl-1 as well as A1 do not contain sequences at the N-terminal region that encompass the BH4 domain [103]. This domain is found in anti-apoptotic proteins Bcl-2 and Bcl-X_L and has been shown to be essential for the anti-apoptotic activity of these proteins[110]. Nevertheless Mcl-1 has been proposed as an apoptotic protein based on experiments involving the transfection or overexpression of *mcl-1* [111, 112].

Antisense oligonucleotide inhibition has been use to further delineate the role of Mcl-1 in apoptosis [113]. An oligonucleotide was designed to target the *mcl-1* mRNA of human polymorphonuclear leukocytes. This ASO was designed with a partial phosphorothioate backbone and modified 5' and 3' end nucleotides to render it resistant to nucleases. Results of this work showed that Mcl-1 is essential to the delay of apoptosis during the aging of human polymorphonuclear leukocytes in culture [113].

In other studies, phosphodiester and chimeric methylphosphonate/phosphodiester oligonucleotides were used to denote the role of Mcl-1 as an important anti-apoptotic factor in a differentiating human myeloblastic leukemia cell line [114]. The ASOs effectively depleted Mcl-1 protein without affecting several other Bcl-2 family members, i.e., Bcl-2, Bak, and Bax. Inhibition of Mcl-1 resulted in a rapid progression to apoptosis. Importantly, i was determined that Bcl-2, which is constitutivel expressed in these cells, did not prevent apoptosi with Mcl-1 protein depletion. The expression o Bak, a pro-apoptotic protein, increases durin differentiation of myeloblastic cells int monocytes. It has been hypothesized that increas in Bak may be responsible for the onset o apoptosis. This study also supports a previous report using plasmid- encoded antisense construct in differentiating myeloid leukemia cells where ar apoptotic role of Mcl-1 was suggested [115]. As mentioned above with breast cancer cells, anti- and pro- apoptotic proteins of the Bcl-2 family proteins have complex and interrelated roles in apoptosis of leukemia cells.

CONCLUSIONS

New oligonucleotide analogs are being developed to further increase the activity and specificity of antisense compounds, and also to minimize toxicity. The important aspect of ASO delivery is also being explored and new methods of delivery need to be tested in vivo. These developments are crucial to the improvement of the therapeutic value of ASOs in the treatment of cancers and other diseases, especially since there are mixed results in current clinical trials using available ASO biotechnology.

A current cancer research focus involves the inhibition of anti-apoptotic gene expression via antisense oligonucleotides. Cancer cells have a complex expression of anti- and pro-apoptotic genes. Antisense studies are contributing to the understanding of the relative importance of Bcl-2 family member proteins to apoptosis of tumor cells, such as lung cancer cells. Experiments involving the inhibition of *bcl-x_L* can lead to new clinical trials with cancers using *bcl-x_L* mRNA as an antisense target. Antisense oligonucleotides directed against anti-apoptotic proteins may also increase chemosensitization of tumors. Notable are the results with prostate cancer cells and ASO/paclitaxel treatment, colon cancer and ASO/5-fluorouracil, and malignant melanoma where current clinical trials using the ASO/dacarbazine combination for treatment

appear very encouraging. In the development of ASOs as anti-cancer drugs, novel techniques are being tried such as use of bispecific oligonucleotides or the simultaneous administration of multiple ASO against multiple anti-apoptotic gene targets in tumor cells. If these methods continue to show success they can form the basis for future clinical trials using two or more gene targets.

Future preclinical experiments should include microarray gene expression analysis to ascertain whether changes occur in expression of proteins other that of the targeted protein with exposure of cells to a specific ASO. These controls are needed to verify the specificity of an ASO.

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